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The overall objective of my research project, was to define the biological function of the mouse *Notch4* gene. Based on genetic analysis in *Drosophila* and *C. elegans,* Notch proteins have been demonstrated to regulate cell fate decisions during development. Using *in vitro* and *in vivo* models, my work has provided evidence that supports the model that Notch4 modulates cell fate decisions in the mouse mammary gland and developing endothelium. Branching morphogenesis of the mouse mammary epithelial TAC-2 cell line was used as an assay to examine the role of Wnt, HGF, TGF-β and the Notch receptors in branching morphogenesis. Rat brain micro vessel endothelial cells were used as a model system to study the role of Notch4 and Jagged-1, a putative Notch ligand, in endothelial cell differentiation. To study the *in vivo* role of Notch4 in endothelial cell development, homologous recombination in embryonic stem cells was used to drive expression of *Notch4* to endothelial cells during embryonic development. Activating mutations in the *Notch4* gene disrupt the normal architectural patterns that are required for proper development of these tissues. These observations support a role for Notch signaling in cell fate determination during mammary gland and endothelial cell differentiation.

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Chapter 1

Introduction

Notch proteins

The Notch family of proteins consists of several members that are found in distantly related organisms. The prototypes are *Drosophila* Notch (Wharton et al., 1985). and *C. elegans* lin-12 (Greenwald, 1985; Yochem et al., 1988) and glp-1 (Yochem and Greenwald, 1989). In recent years, several homologues of Notch have been identified in vertebrates, including zebrafish, frogs, mice, rat, goldfish, chick and humans. In mice four Notch genes have so far been identified (Lardelli et al., 1994; Lardelli and Lendahl, 1993; Uyttendaele et al., 1996).

All Notch/lin-12 family members encode for transmembrane receptor proteins containing a signal peptide, an extracellular domain consisting of a series of repeated motifs, a membrane spanning sequence, and an intracellular domain consisting of several repeated motifs. The predicted extracellular domain of Notch/lin-12 family members consists of a variable number of EGF (Epidermal Growth Factor-like) repeats, as well as several cysteine-rich repeats referred to as Notch /lin-12 repeats. For instance, *Drosophila* Notch encodes 36 EGF repeats in a tandem array followed by 3 Notch/lin-12 repeats. The intracellular domains of Notch/lin-12 proteins contains a tandem array of an approximately 40 amino acid motif that has been termed the cdc10/SW16-repeat (Aves et al., 1985; Breeden and Nasmyth, 1987) or sometimes the ankyrin repeats (Lux et al., 1990). This repeated motif is believed to mediate protein-protein interactions and is found in various distantly related proteins, such as cell cycle-regulatory proteins and DNA binding proteins. The intracellular domain of Notch/lin-12 proteins also contain a cluster of proline, glutamic acid, serine, and threonine residues (referred to as PEST sequences) that are thought to be targets for proteolysis or may also represent potential phosphorylation sites (Rogers et al., 1986). Finally, the intracellular domain of some Notch/lin-12 proteins contains an opa domain, which is rich in glutamine and is commonly found in transactivating domains of transcription factors or transcription factor

binding proteins. It is of note that the intracellular domain of Notch/lin-12 proteins do not encode serine/threonine- or tyrosine-kinase domains, as is often found in cell surface signaling receptors. Thus, it is thought that signaling through Notch/lin-12 proteins is carried out by novel intracellular signaling pathways.

Notch ligands

Genetic and molecular analyses have identified several Notch ligands in *Drosophila* and *C. elegans*. The *Drosophila Delta* and *Serrate* genes and *C. elegans lag-2* and *apx-1* genes encode a family of structurally related ligands for the *Drosophila* Notch and the *C. elegans* lin-12 and glp-1 receptors (Austin et al., 1995; Ball et al., 1988). These ligands are all membrane bound proteins of which the extracellular domains contain a variable number of EGF-like repeats and a cysteine-rich DSL (Delta-Serrate-Lag-2) motif. Although in general these structural motifs are believed to participate in ligand-receptor interaction, it is not clear what specific regions are involved in receptor binding and how they interact with the receptor.

Notch ligands have also been identified in vertebrates. *Jagged* , a rat homologue of Serrate, was cloned from Schwann cell cDNA library by low stringency hybridization (Lindsell et al., 1995). Another putative mouse Notch ligand *Dll1* (Delta-like 1) has been cloned and shown to be closely related to *Drosophila* Delta (Hrabe de Angelis et al., 1997). **It is unclear if the different Notch ligands display specific affinities towards different Notch proteins. Instead, it appears that different ligands are functionally interchangable. For instance, *C. elegans* apx-1 can fully substitute for lag-2 when expressed under the control of lag-2 regulatory sequences (Fitzgerald and Greenwald,** 1995). Similarly, *Drosophila* Serrate can functionally replace Delta during neuroblast segregation in the *Drosophila* embryo (Fleming et al., 1990).

Notch signalling

Genetic and molecular studies have also identified intracellular components of the Notch signaling pathway. The best characterized signaling molecule is the *Drosophila* gene *Suppressor of Hairless (Su(H))*, which was isolated in a genetic screen for mutations that attenuate Notch signalling in the developing *Drosophila* eye (Fortini and Artavanis-Tsakonas, 1994) and is required in sensory organ development in *Drosophila* (Schweisguth and Posakony, 1992). It encodes for a protein that is homologous to the mammalian transcription factor RBP-Jk (recombination signal sequence binding protein for Jk genes) or CBF-1 (C-promoter binding factor 1). The RAM23 domain which is localized between the transmembrane and cdc10 repeats of Notch has been demonstrated to be the binding site of Su(H) (Hsieh et al., 1996). Su(H) protein is translocated to the nucleus when Notch binds to its ligand Delta (Simpson et al., 1992) where it can activate the transcription of target genes. Genetic studies have shown that *Hairless*, which encodes a novel basic protein, can antagonizes Su(H) activity. Consistent with this genetic relationship, Hairless can bind *in vitro* to Su(H) and prevent the transcription factor from binding to DNA (Brou et al., 1994). Su(H) activates the expression of the *Enhancer of Split* genes, which encode for basic Helix-Loop-Helix (bHLH) proteins (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). In *Drosophila*, *Enhancer of Split* genes downregulate the transcription of another family of bHLH genes, the *achaete/scute (ac/sc)* genes (Heitzler et al., 1996; Oellers et al., 1994). Hence, the end result of Notch activation is the downregulation of the *ac/sc* genes. In Drosophila, the presence or absence of the *ac/sc* proteins determines the cell fate. For instance, loss of Notch leads to expression of *ac/sc* in all cells and consequently all cells adopt the neural fate, leading to the lethal hypertrophy of the nervous system. Conversely, constitutive activated forms of Notch lead to downregulation of ac/sc and all cells adopt the epidermal fate. Vertebrate homologues of *Enhancer of Split* and *ac/sc* genes have been isolated: HES-1(Jarriault et al., 1995) and MASH-1(Ishibashi et al., 1995)

respectively. It has been demonstrated that CBF1, a Su(H) homologue, activates transcription of HES-1, *Enhancer of Split* homologue, and HES-1 in turn down-regulates MASH-1, a homologue of *ac/sc* (Ishibashi et al., 1995). Therefore, in both flies and vertebrates, conservation embraces not only the core components, but also downstream target genes and their targets.

Several other proteins have been identified that can bind to the intracellular domain of Notch. The region of the lin-12 protein that includes the RAM-23 domain and cdc10 repeats appears to interact with a downstream and positive regulator, EMB-5 (Hubbard et al., 1996). Deltex, a novel cytoplasmic protein, has been demonstrated to bind to the cdc10 repeats of Notch (Diederich et al., 1994). Genetic analysis demonstrated that Deltex is a positive regulator of Notch, since overexpression of Deltex and activated Notch produce similar phenotypes in Drosospihla. However it appears to act upstream of Notch signalling since activated Notch can rescue deltex loss of function mutations(Artavanis-Tsakonas et al., 1995). More recently, Sel-10, a member of the CDC4 family of proteins, has been identified and shown to form a complex with lin-12 or murine Notch4 (Hubbard et al., 1997). sel-10 is a negative regulator of lin-12/Notch-mediated signaling in C. elegans.

Notch and cell fate decisions

The normal functions of *Notch/lin-12* genes have been analyzed in several organisms; most extensively in those tractable to genetic or biochemical analysis of early development. These studies demonstrated that the Notch and lin-12 proteins are required for cell-cell interactions that play a pivotal role in cell-fate decisions. For instance, the mechanisms that control how a group of equivalent progenitor cells give rise to a group of cells each with their particular fate. This function is of course of fundamental importance during development as was demonstrated with *lin-12*, *Notch* and *Xotch*

mutants. Mutations consisting of deletion of the extracellular domain of lin-12(Struhl et al., 1993) or Notch (Fortini et al., 1993; Lieber et al., 1993; Struhl et al., 1993) results in a dominate gain-of-function phenotype. The truncated gene products encoding for the intracellular part of the proteins behave as a constitutively active version of the normal protein. Thus, the Notch/lin-12 intracellular domains have an intrinsic activity similar to that of the activated intact protein. Genetic mosaic analysis in *C. elegans* and *Drosophila* demonstrated that Notch functions as a receptor for intercellular signals (Heitzler and Simpson, 1991; Seydoux and Greenwald, 1989). In the retina of *Xenopus* and *Chick*, Notch signalling restricts the number of cells which can progress along their differentiation pathway. The function of Notch in regulating cell fate decisions is also present in mammals. In the mouse developing immune system, Notch1 has been implicated in the CD8/CD4 T cell lineage decision (Robey et al., 1996). Thus, in each system where the function of Notch has been studied, Notch acts by restricting cell fates or by allowing only some cells to respond to fate-determining signals.

Notch and tumorigenesis

By analogy to the function of Notch/lin-12 family members, one could hypothesize that vertebrate *Notch* genes may control the choice between a stem cell fate and a more differentiated fate. Disruption of this decision by *Notch* mutations could cause both daughter cells to adopt the stem cell fate. This would then lead to a delay in differentiation and accumulation of pluripotent highly proliferative stem cells, thereby increasing the probability for secondary oncogenic mutations, and ultimately leading to the development of tumors. Evidence for the involvement of *Notch* mutations in tumorigenesis comes from the analysis of the mouse *int-3* gene and human *Notch1* gene.

The *int-3* gene was originally identified based on its oncogenic effects in the mouse mammary gland (Gallahan et al., 1987; Robbins et al., 1992) . This gene was found to be

a frequent target for insertional activation by mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary gland tumors (Gallahan et al., 1987). In Czech II mice, about 20% of the MMTV induced tumors contain an *int-3* gene into which the MMTV viral genome has been inserted (Gallahan and Callahan, 1987). Viral integration results in expression of the 2.4 kb int-3 transcript (Joutel et al., 1996). The cloned *int-3* gene fragment encodes a truncated protein that is highly homologous to the intracellular part of the lin-12/Notch protein family. Therefore, it was believed that the *int-3* gene corresponds to a truncated form (i.e. intracellular domain) of a novel mouse *Notch* gene, which would encode for a transmembrane receptor structurally similar to Notch/lin-12 proteins. Work described in Chapter 3 confirms and extends this inference. Transgenic mice that express the truncated int-3 develop poorly differentiated mammary adenocarcinomas at 100% penetrance. Expression of the *int-3* gene, under the control of MMTV LTR, in the mammary gland of transgenic mice reveals a complex phenotype, suggesting that ectopic expression of the *int-3* oncogene causes deregulation of normal developmental controls and hyperproliferation of glandular epithelia (Jhappan et al., 1992). First, mammary glands of transgenic mice were arrested in development and were lactation deficient. Second, focal and multiple poorly differentiated mammary adenocarcinomas appeared in the majority of transgenic mice between 2 and 7 months of age. Since expression of the *int-3* oncogene leads to incomplete differentiation and proliferation of immature epithelial cells it has been proposed that int-3 induces oncogenesis by diverting cell fate into a proliferating stem cell of the mammary epithelium. These proliferating cells display a growth advantage and are subject to other mutational events, ultimately resulting in a tumor. Finally, expression of the *int-3* gene in an established mammary epithelial cell line, HC11(Ball et al., 1988) induces anchorage-independent growth in soft agar. These studies have established that the *int-3* gene is a potent oncogene in mouse mammary tumorigenesis.

The human *Notch1* gene, *TAN-1*, was defined by multiple independently isolated translocation breakpoints in patients with acute T lymphoblastic leukemia. Similar to the *int-3* activation by MMTV integration, the chromosomal translocations results in the transcription of the truncated *TAN-1* gene (Ellisen et al., 1991). This truncated TAN-1 product has also been shown to promote T cell neoplasm in bone marrow reconstitution assays (Pear et al., 1996) . Furthermore, human *Notch1* and *Notch2* were found to be overexpressed in human cervical cancers (Zagouras et al., 1995). These data thus clearly indicate the involvement of *Notch* genes in tumorigenesis.

Chapter 2

Materials and Methods

Isolation and sequencing of *Notch4* cDNA clones

A 1680 bp fragment was amplified by PCR from adult mouse testis cDNA (RT-PCR) using specific primers (5' primer: CGTCCTGCTGCGCTTCCTTGCA and 3' primer CCGGTGCCTAGTTCAGATTTCTTA) designed from the *int-3* cDNA sequence (Robbins et al., 1992). This cDNA fragment corresponds to the previously cloned *int-3* oncogene. Two consecutive 5' RACE reactions (5'-Amplifinder RACE kit, Clonetech) using testis and lung cDNA were done to obtain cDNA clones located 5' of the *int-3* oncogene. The above described cDNAs were cloned into Bluescript KS (Stratagene) and the TA cloning vector (Invitrogen) and used to generate probes to screen a lung cDNA library (Clonetech). Briefly, nitrocellulose membranes (Schleicher&Schuell) were hybridized in a solution containing 50% Formamide, 3x SSC, 100 mM Tris-HCl (pH 7.4), 5x Denhardt's solution, 0.2% SDS, and 0.1 mg/ml salmon sperm DNA at 42°C for 14 hours. Filters were then washed in 1x SSC and 0.5% SDS at room temperature followed by washes at 65°C. Positive clones were purified and sequenced to confirm overlapping regions. Novel 5' restriction fragments of these newly isolated clones were used in consecutive screens in order to obtain the full length *Notch4* cDNA. All the above described clones were sequenced using the dideoxy termination method (Sanger) with an automatic DNA sequencer (Applied Biosystems). Sequence data from both strands were obtained for the entire *Notch4* cDNA and were analyzed and assembled using computer software (MacVector, Assemblylign).

Northern Blot analysis

Total RNA was isolated from adult CD-1 mouse tissues and Northern blot hybridization analysis was performed. 20 mg of total RNA was electrophoresed on a 1% agarose gel containing 6% formaldehyde. After electrophoresis RNAs were transferred to a nylon membrane (Duralon-UV membranes, Stratagene) by capillary blotting. ^{32}P-radiolabeled riboprobes were transcribed (Maxiscript *in vitro* transcription kit, Ambion) from *Notch4*

cDNA clones encoding the 5' or 3' UTR (untranslated region) or ORF (open reading frame). The 3' UTR *Notch4* cDNA clone was isolated by RT-PCR and a 440 bp restriction fragment of this cDNA was used as riboprobe. Hybridization solution contained 60% Formamide, 5x SSC, 5x Denhardt's solution, 1% SDS, 20 mM NaH_2PO4 (pH 6.8), 0.1 mg/ml salmon sperm DNA, 100 ug/ml yeast tRNA, 10 ug/ml poly-A mRNA and 7% dextran sulfate and was done for 14 hours at 65°C. Washing solution contained 2x SSC and 1% SDS and was done at room temperature and 50°C for 15 minutes each, followed by a 2 hour wash at 80°C with a solution containing 0.2x SSC and 1% SDS. Membranes were exposed on X-ray film (X-OMAT AR, Kodak). The integrity of the RNA, as well as comparable amounts of RNA were tested by rehybridization with a *GAPDH* probe. Total RNA was isolated from RBE4 cell lines and Northern blot hybridization analysis was performed as described above. ^{32}P-radiolabeled riboprobes were transcribed (Maxiscript *in vitro* transcription kit, Ambion) from *Notch1*, *Notch3*, *Notch4* and *Jagged-1* cDNA clones. The integrity of the RNA, as well as comparable amounts of RNA were tested by re-hybridization of blots with a β-actin probe.

In situ hybridization

Staged embryos ranging from 9 days to birth were obtained from timed breedings of CD-1 mice. Morning of the vaginal plugs was counted as 0.5 days post coitum (p.c.). Preparation of tissue and subsequent procedures for in situ hybridization were done as previously described (Marazzi and Buckley, 1993; Sassoon and Rosenthal, 1993). After hybridization and procedures, sections were dehydrated rapidly and processed for standard autoradiography using NTB-2 Kodak emulsion and exposed for two weeks at 4°C. Analysis was carried out using both light and dark field optics on a Leica DA microscope. To avoid potential cross-hybridization with homologous RNAs, we used an

antisense ^{35}S-labeled RNA probe corresponding to the 3' UTR of *Notch4* . Probes were used at a final concentration of $9x10^4$ dpm/ml.

Reagents

Recombinant human hepatocyte growth factor (HGF) was provided by Genentech, Inc. (San Francisco, CA). Recombinant TGF-β2 was provided by Dr. G. Gunderson (Columbia University, New York, NY). Recombinant human basic fibroblast growth factor (bFGF) was provided by Dr. Anthony Belve (Columbia University, New York). Rat tail collagen solution was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-HA monoclonal antibody (12CA5) was from Berkeley Antibody Co. (Richmond, CA), HRP-conjugated sheep anti-mouse immunoglobulin G from Amersham (Arlington Heights, IL), and rat anti-mouse CD 31 PECAM-1 was obtained from PharMingen. Restriction and DNA modifying enzymes were obtained from New England Biolab Inc. and reagents for histochemical analysis of γGTP and ALP activity from Sigma.

cDNA clones

The murine *Notch4/int-3* cDNA corresponds to a truncated *Notch4* cDNA; residues 4551 to 6244 of *Notch4* (Uyttendaele et al. 1996). An oligonucleotide encoding the haemagglutinin (HA) antigenic determinant was appended to the 3' end of the *Notch4/int-3* , *Notch4* and*Wnt*1 cDNA's. Eighteen codons were added that specify the amino acid sequence SMAYPYDVPDYASLGPGP, including the nine residue HA epitope (underlined). HA-tagged *Notch4/int-3, Notch4* and*Wnt*1 cDNAs were created by subcloning each cDNA into Bluescript KS (Stratagene) with the coding region of the HA epitope situated downstream of the newly inserted cDNA. These two sequences were made co-linear by "loop-out" mutagenesis using oligonucleotides designed to eliminate the stop codon and non-coding 3' sequence of the *Notch4/int-3* and *Wnt*1 cDNAs.

Oligonucleotides used in this procedure are as follows; *Notch4/int-3*: CGG TTG TAA GAA ATC TGA ACT CCA TGG CCT ACC CAT ATG, *Wnt*-1: CGC GCG TTC TGC ACG AGT GTC TAT CCA TGG CCT ACC C . The 5' end of each oligo is complementary to the C-terminus of *Notch4/int-3* or *Wnt*1 cDNA and their 3' ends anneal to HA epitope-encoding sequence (underlined). Mutagenesis was done with the Muta-Gene*in vitro* mutagenesis kit (Bio Rad, Richmond, CA). The presence of each fusion was confirmed by DNA sequencing. *Notch4/int-3* cDNA deletion mutants were generated from the epitope-tagged *Notch4/int-3* construct by restriction enzyme cloning, and were named ΔNT, ΔCDC, ΔCT and ΔNTΔCT. The ΔNT deletion mutant corresponds to nucleotides 4921 to 6244 of the *Notch4* sequence. The ΔCDC deletion mutant corresponds to nucleotides 4551 to 4864 and to nucleotides 5706 to 6244 of the *Notch4* sequence. The ΔCT deletion mutant corresponds to nucleotides 4551 to 5718 of the *Notch4* sequence. The ΔNTΔCT deletion mutant corresponds to nucleotides 4921 to 5718 of the *Notch4* sequence. The *Jagged-1* cDNA, HA-tagged at the carboxy terminus, was obtained from Dr. Gerry Weinmaster (UCSF, California).

Cell culture

The TAC-2 cell line was derived from NMuMG cells as described previously (Soriano et al., 1995). TAC-2 cell were grown on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO). The Bosc 23 retrovirus packaging cell line (Pear et al. 1993) was obtained from Dr. W. Pear (MIT, MA) and grown in DMEM containing 10% FCS. Both culture media were supplemented with penicillin (500 i.u./ml) and streptomycin (100 υg/ml). Rat brain microvessel endothelial cells (RBE4) cells were plated on collagen coated dishes and maintained in Alpha MEM/Ham's F10 (1:1) supplemented with 2 mM glutamine, 10% fetal calf serum and penicillin/streptomycin. Medium was changed twice a week, and cells were passaged at a split ratio of 1:4. All cell lines were grown at 37°C in 8% CO_2.

Cell line generation

HA-tagged cDNAs were inserted into the retroviral vector pLNCX (Miller and Rosman, 1989) wherein neomycin phosphotransferase (neo) expression is controlled by the murine leukemia virus LTR, and cDNA transcription is controlled by the cytomegalovirus (CMV) enhancer/promoter. The retroviral vector pLHTCX was derived from pLNCX, however the neo gene is replaced by a hygromycin-resistence gene. Populations of TAC-2 cells, expressing either HA-tagged *Notch4/int-3* or *Wnt*1 cDNA, were prepared by retroviral infection. Recombinant retroviruses were generated by transiently transfecting constructs into the BOSC 23 cell line by calcium phosphate co-precipitation, as previously described (Pear et al., 1993). Retroviral infection of TAC-2 cells was done by culturing cells with viral supernatants collected from transfected BOSC 23 cells two day post-transfection. Infections were done in the presence of 4 μg/ml polybrene for 12 hours after which medium was replaced to DMEM + 10% FCS. One day post-infection the culture medium was replaced to DMEM + 10% FCS containing 500 μg/ml Geneticin (GIBCO BRL Life Technologies, Grand Island, NY) or 200 μg/ml hygromycin B (Sigma Chemical Co.). Colonies appeared 5 days later and were pooled into medium containing 250 μg/ml Geneticin or 200 μg/ml hygromycin B. These resultant populations, each comprised of at least 50 clones, were used in assays described below. HA-tagged cDNAs (Notch4/int-3, Jagged-1, and Notch4) were inserted into the retroviral vector pLHTCX wherein hygromycin-resistence/thymidine kinase fusion gene phosphotransferase gene expression is controlled by the murine leukemia virus (MLV) long terminal repeat (LTR), and cDNA transcription is controlled by an internal cytomegalovirus (CMV) enhancer/promoter. Distinct populations of RBE4 cells, either expressing Notch4/int-3 or Jagged-1 cDNA, were prepared by retroviral infection as described above. One day post-infection the culture medium was replaced to medium containing 100 μg/ml hygromycin B (Sigma). Colonies appeared 5 days later and were pooled. The resultant populations,

each comprised of at least 50 clones, were used in cellular and biochemical assays described below. Since the Notch4 cDNA is too large to be accommodated in retroviral vectors due to retroviral packaging constraints, Notch4 expressing cell lines were established by transfecting RBE4 cells directly (calcium phosphate transfection).

Collagen cell culture assays

TAC-2 cell lines were harvested using trypsin-EDTA, centrifuged, and embedded in three-dimensional collagen gels as previously described (Soriano et al., 1995). Briefly, 8 volumes of rat tail collagen solution (approximately 1.5 mg/ml) were mixed with 1 volume of 10x minimal essential medium (GIBCO) and 1 volume of sodium bicarbonate (11.76 mg/ml) in a sterile tube kept on ice to prevent premature collagen gellation. TAC-2 cells were resuspended in the cold mixture at cell densities of 2 or 4 x 10^4 cells/ml and 0.5 ml aliquots were dispensed into 16-mm wells of 24 multiwell plates (Becton Dickinson Labware). After the collagen mixture had gelled, 1ml of complete medium (DMEM + 10% FCS) with or without HGF or TGF-β2 was added to each well. TAC-2 collagen gel cultures were initially done in the presence and absence of 2mM sodium butyrate, but since no difference in phenotypes was observed, the sodium butyrate was omitted in all experiments. Media were changed every 2 days, and after 3 to 5 days, cell cultures were photographed with a Nikon ELWD 0.3 phase contrast microscope on Kodak T-Max film (100 X magnification).

Quantification of cord length and branching

TAC-2 cells were suspended at 5x10^3 or 1x10^4 cells/ml in collagen gels (500 µl) cast into 16-mm wells of 4-well plates (Nunc, Kampstrup, Rosklide, Denmark) and incubated in 500 µl complete medium in the presence or the absence of 10ng/ml HGF. After 7 days, the cultures were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, and at least 3 randomly selected fields (measuring 2.2 mm x 3.4 mm) per experimental

condition in each of 3 separate experiments were photographed with a Nikon Diaphot TMD inverted photomicroscope. The total length of the cords present in each individual colony was measured with a Qmet 500 image analyzer (Leyca Cambridge Ltd., Cambridge, UK). Cord length was considered as "0" in: a) colonies with a spheroidal shape, and b) slightly elongated structures in which the length to diameter ratio was less than 2. Quantification of branching was performed by counting all branch points in each colony. Values of cord length and branching obtained from the largest colonies are an underestimate, since in these colonies a considerable proportion of cords were out of focus and therefore could not be measured. Values were expressed either as mean cord length and number of branch points per photographic field (Soriano et al., 1996) or as mean cord length and number of branch points per individual colony (Soriano et al., 1995). The mean values for each experimental condition were compared to controls using the Student's unpaired T-test.

Immunoblot analysis

HA-tagged Notch4/int-3, Notch4/int-3 deletion mutants and Wnt-1 proteins from lysates of TAC-2 cell populations were analyzed by immunoblotting. To maximize protein expression, TAC-2 cells were treated with 2mM sodium butyrate for 16 hours prior to lysis. Cells were washed twice with cold PBS and then removed from dishes in 1.5 ml PBS using a rubber policeman. Cells were pelleted by centrifugation at 2,000x g at 4°C for 5 min. and lysed in 90 µl TENT buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton-X100) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin, at 4°C for 30 min. Lysates were clarified by centrifugation at 10000xg at 4°C for 10 min., and protein contents were determined using the BioRad Protein determination kit. Lysates containing 40 µg protein were electrophoresed in 10% SDS-polyacrylamide gels. Proteins were transferred from gels onto nitrocellulose by electroblotting, and then blocked overnight at 4°C in TBST

(10 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween-20) containing 1% bovine serum albumin (fraction V). Blots were then incubated in anti-HA monoclonal antibody (12CA5) diluted 1:100 in TBST at room temperature. After four hours, the blot was washed three times for 5 min. each in TBST. Blots were exposed to a 1:16,000 dilution of HRP-conjugated sheep anti-mouse IgG. Blots were washed as above and then incubated 1-2 min. in enhanced chemiluminescence reagents (Amersham Inc, IL) and exposed to X-ray film (Fujifilm, Fuji Photo Film Co., LTD., Tokyo). HA epitope-tagged or non-tagged Notch4/int-3, Jagged and Notch4 proteins from lysates of RBE4 cells were analyzed by immunoblotting as described above. The anti-mouse Notch4 antiserum was used at 1:2000 dilution, this antibody is directed against the carboxy-terminal region of Notch4 (residues 1788-1964).

Immunohistochemistry

Frozen, non-fixed, adult mouse kidney sections were post-fixed in 100 % acetone at -20^0 C and rinsed in 0.1% Triton X-100 in PBS (PBST). Tissue sections were either incubated in 0.3% peroxidase in PBS for 20 minutes (horse radish peroxidase detection) or were incubated in 20% acetic acid for 15 seconds (alkaline phosphatase detection). The sections were rinsed twice in PBST and were incubated in blocking solution (5% normal goat serum, 3% bovine serum albumine in PBS) for 1 hour. Excess blocking solution was removed and sections were covered with 1^0 antibody in dilution solution (1% normal goat serum, 3% bovine serum albumine in PBS) for 12 hours at 4^0 C. The anti-mouse CD 31 antibody was used at a final concentration of 3 microgram per ml, the anti-mouse Notch4 rabbit immune-serum and the rabbit pre-immune serum were diluted 500 fold. Tissue sections were rinsed 4 times for 10 minutes in PBST. Secondary antibodies (biotinylated goat anti-rat IgG and goat anti-rabbit IgG labeled to alkaline phophatase, both at 1:200 dilutions) were applied in dilution buffer for 2 hours at room temperature. Tissue sections were rinsed 4 times for 10 minutes in PBST. Biotinylated goat anti-rat

IgG was detected by the peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories), and alkaline phosphatase labeled goat anti-rabbit IgG was detected as previously described. Reactions were stopped by rinsing in water, for alkaline phospatase staining sections were counterstained with eosine, dehydrated through increased alcohol series and xylene and mounted.

RBE4 microvessel outgrowth assay

For microvessel outgrowth assay, RBE4 cell lines were plated at equal cell densities (50% confluence at plating). After 3 to 5 days, cell cultures were photographed with a Nikon ELWD 0.3 phase contrast microscope (100 X magnification).

Histochemical enzyme assay

γGTP activity was demonstrated histochemically in cell cultures fixed in acetone, as described previously: L-gamma-glutamyl-4-methoxy-2-naphthylamide was used as substrate and glycyl-glycine as acceptor. In a simultaneous azo coupling reaction with Fastblue BB, a red azo dye is produced. ALP activity was determined in cell cultures fixed in citrate-buffered acetone, using the reaction mixture Sigma kit N85, with naphtol AS-MX phosphate as substrate and fast blue RR salt as diazonium salt, as previously described.

***In vitro* differentiation of ES cells**

In vitro **differentiation of ES cells was performed as previously described. Briefly, ES cells were passaged twice on gelatinized plates. On day 6 after the second passage, ES colonies were treated with dispase, washed and grown in suspension in bacterial dishes in ES media without LIF. For attached embryoid body cultures, embryoid bodies were transferred to tissue culture plates on day 3 post-dispase treatment. On day 8 post-dispase treatment, β-galactosidase activity was detected as previously described.**

Chapter 3

Notch4/int-3, a mammary proto-oncogene, is an endothelial cell specific mammalian *Notch* gene

Introduction

The *int-3* gene was originally identified based on its oncogenic effects in the mouse mammary gland. *int-3* is a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (Gallahan et al., 1987; Robbins et al., 1992; Sarkar et al., 1994). Tumor specific transcripts derived from the *int-3* gene encode a protein homologous to the intracellular part of the Notch family of cell surface receptors. Exogenous expression of the int-3 oncoprotein has been shown to affect the growth and development of mammary epithelial cells. Overexpression of the int-3 oncoprotein in a mouse mammary epithelial cells (HC11) promotes anchorage independent growth (Robbins et al., 1992). Expression of *int-3* as an MMTV-LTR driven transgene in the mouse mammary gland results in abnormal development of the mammary gland and rapid development of undifferentiated mammary carcinomas (Jhappan et al., 1992). In the normal mouse mammary gland, endogenous int-3 protein has been detected in mammary stroma and epithelium (Smith et al., 1995).

Members of the *Notch/lin-12* gene family were first identified in *Drosophila* and *Caenorhabditis elegans* through genetic analysis of mutations that alter cell fate decisions (for review see (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992)Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992). *Drosophila Notch* regulates multiple cell fate decisions that involve cell-cell interactions during fly development, for instance, control of cell fate decisions involving neural/epidermal specification in proneural clusters (Artavanis-Tsakonas and Simpson, 1991). The *C. elegans* lin-12 and glp-1 proteins are structurally related to Notch, and are also involved in cell fate specifications during development in the nematode (Greenwald, 1985; Yochem and Greenwald, 1989). Genetic analysis of *Notch/lin-12* genes suggest that this family of

genes controls binary cell fate decisions and inductive signaling that depend on cell-cell interactions (reviewed in Artavanis-Tsakonas et al., 1995; Greenwald, 1994; Greenwald and Rubin, 1992). Alternatively, *Notch/lin-12* genes have been proposed to block cell differentiation, thus maintaining the competence of cells for subsequent cell-fate determination (Coffman et al., 1993; Fortini et al., 1993).

Notch/lin-12 genes encode transmembrane receptor proteins characterized by highly repeated, conserved domains. The amino terminus of Notch proteins encodes the extracellular domain and contains as many as 36 repeats of an EGF-like motif involved in ligand binding (Rebay et al., 1993), and three tandem copies of a Notch/lin-12 sequence motif of unknown function. The intracellular portion of Notch proteins is characterized by six tandem copies of a cdc10/ankyrin motif, thought to be a protein-protein interaction domain (Michaely and Bennett, 1992), and a PEST sequence motif which may represent a protein degradation signal (Rogers et al., 1986). In several systems, truncated forms of Notch/lin-12 proteins that contain an intact intracellular domain without most of the extracellular domain behave as constitutively activated receptors (reviewed in Artavanis-Tsakonas et al., 1995; Greenwald, 1994). The human Notch 1 orthologue, TAN-1, was first identified in independently isolated translocation breakpoints in acute T lymphoblastic leukemia and is predicted to encode a truncated product that has an intact intracellular domain but lacks most of the extracellular domain (Ellisen et al., 1991). Similarly, the int-3 oncoprotein encodes the intracellular domain of a Notch-like protein and thus has been proposed to act as an activated Notch receptor (Robbins et al., 1992).

Based on sequence similarity to *Drosophila Notch*, additional *Notch*-related genes have been isolated from mammals; including mouse (Franco Del Amo et al., 1993; Lardelli et al., 1994; Lardelli and Lendahl, 1993; Reaume et al., 1992), rat (Weinmaster et al., 1992; Weinmaster et al., 1991), and human (Ellisen et al., 1991; Stifani et al., 1992; Sugaya et

al., 1994). To date, three *Notch* homologues, *Notch1*, *Notch2*, and *Notch3*, have been identified in the mouse and their embryonic expression patterns display partially overlapping but distinct patterns of expression that are consistent with a potential role in the formation of the mesoderm, somites, and nervous system (Williams et al., 1995). Abundant expression of *Notch1*, *Notch2*, and *Notch3* is found in proliferating neuroepithelium during central nervous system development. Targeted disruption of the *Notch1* gene in mice results in embryonic death during the second half of gestation (Conlon et al., 1995; Swiatek et al., 1994) and homozygous mutant embryos display delayed somitogenesis as well as widespread cell death, preferentially in neuroepithelium and neurogenic neural crest (Conlon et al., 1995; Swiatek et al., 1994).

The gene products of *Drosophila Delta* (Vassin et al., 1987) and *Serrate* (Fleming et al., 1990), and *C. elegans Lag-2* (Henderson et al., 1994; Tax et al., 1994) and *Apx-1* (Mello et al., 1994) are thought to act as ligands for Notch proteins. In the mouse, the orthologue of *Delta*, referred to a *Dll1* (*Delta-like gene 1*), is expressed during embryonic development in the paraxial mesoderm and nervous system in a pattern similar to that of mouse *Notch1* (Bettenhausen et al., 1995). A murine *Serrate*-related gene named *Jagged* has been identified and is partially co-expressed with murine *Notch genes* in the developing spinal cord (Lindsell et al., 1995)

We report here the identification and expression analysis of a fourth murine *Notch* homologue, which we propose to name *Notch4*, reserving the *int-3* nomenclature for the truncated oncogene. Although the intracellular domain of the int-3 oncoprotein shares homology with the Notch/Lin-12 protein family, we now provide a comparison of the full length Notch4 protein to that of the int-3 oncoprotein. The activated int-3 protein encodes only the transmembrane and intracellular domain of the Notch4 protein. The predicted amino acid sequence of Notch4 contains the conserved features of all Notch proteins, however Notch4 has 7 fewer EGF-like repeats compared to Notch1 and Notch2

and contains a significantly shorter intracellular domain. Notch4 is expressed primarily in embryonic endothelium and in adult endothelium and male germ cells.

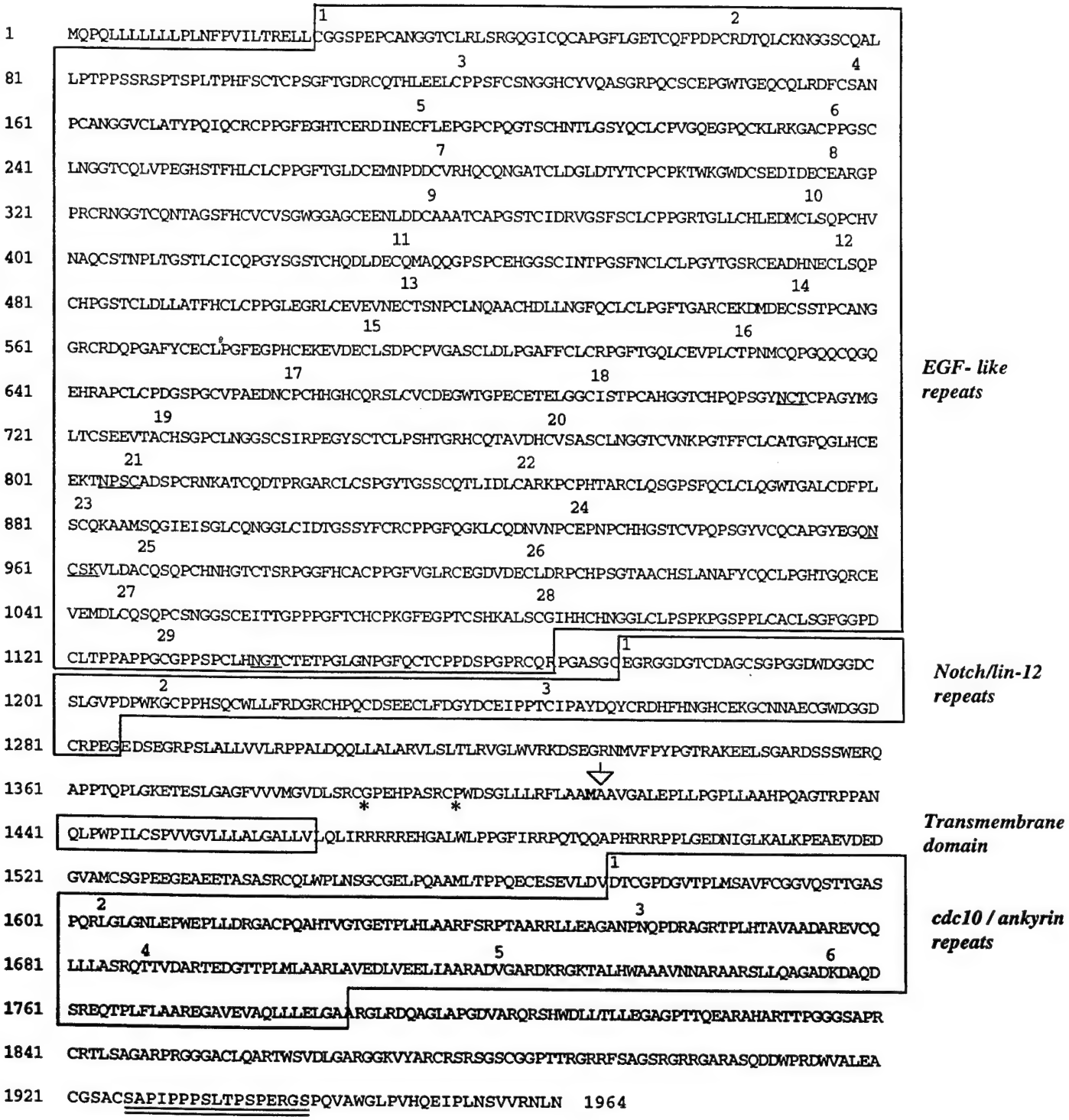
Results

Isolation and Analysis of *Notch4* cDNA clones

The *int-3* mammary oncogene encodes a truncated protein that is highly homologous to the intracellular part of the Notch receptor proteins. The full length *int-3* gene, which we will refer to as *Notch4*, had been proposed to encode a novel member of the Notch protein family (Robbins et al., 1992). To prove this hypothesis, we have cloned cDNAs containing the complete coding potential of the *Notch4* gene. Using primers derived from the published sequence of the *int-3* oncogene, RT-PCR was used to isolate a 2.4 kb *int-3* cDNA encoding the putative intracellular portion of the receptor. To obtain cDNA clones encompassing the full coding potential of the normal *int-3* gene, cDNAs were isolated by 5' RACE and by screening a mouse lung cDNA library. A total of 37 overlapping cDNA clones were analyzed and sequenced to obtain a 6677 bp cDNA sequence. This sequence encodes one long open reading frame of 1964 amino acids, starting with an initiator methionine at nucleotide 347 and terminating with a stop codon at nucleotide 6239. The 6677 bp cDNA corresponds in size to that of *Notch4* transcripts detected by Northern blot analysis; thus, we believe the cloned cDNA represents the full length *Notch4* gene.

Several differences (insertions, deletions, and single nucleotide changes) were found between the nucleotide sequence of *Notch4* reported here and the previously published *int-3* nucleotide sequence (Robbins et al., 1992). These differences alter the reading frame in several locations within the intracellular domain and may be a result of differences in sequence analysis or possibly mutations found in the tumor derived *int-3* transcript (Robbins et al., 1992) that are not found in the *Notch4* gene. The nucleotide sequence of mouse *Notch4* has been deposited with Genbank under the Accession number U43691.

Fig. 1 Deduced amino acid sequence of Notch4 (Genbank Accession number U43691). The boxed regions indicate the major structural elements of the Notch family of proteins, indicated as follows: 29 epidermal growth factor(EGF)-like repeats; 3 Notch/lin12 repeats; Transmembrane domain; 6 cdc10/ankyrin repeats. Putative glycosylation sites are underlined. A putative PEST domain is doubly underlined. The two cysteines thought to promote dimerization are marked with asterisks. The initiating methionine of the int-3 oncoprotein is in bold and marked by an arrow.


1 MQPQLLLLLLLLPLNFPVILTRELLCGGSPEPCANGGTCLRLSRGQGICQCAPGFLGETCQFPDPCRDTQLCKNGGSCQAL
81 LPTPPSSRSPTSPLTPHFSCTCPSGFTGDRCQTHLEELCPPSFCSNGGHCYVQASGRPQCSCEPGWTGEQCQLRDFCSAN
161 PCANGGVCLATYPQIQCRCPPGFEGHTCERDINECFLEPGPCPQGTSCHNTLGSYQCLCPVGQEGPQCKLRKGACPPGSC
241 LNGGTCQLVPEGHSTFHLCLCPPGFTGLDCEMNPDDCVRHQCQNGATCLDGLDTYTCPCPKTWKGWDCSEDIDECEARGP
321 PRCRNGGTCQNTAGSFHCVCVSGWGGAGCEENLDDCAAATCAPGSTCIDRVGSFSCLCPPGRTGLLCHLEDMCLSQPCHV
401 NAQCSTNPLTGSTLCICQPGYSGSTCHQDLDECQMAQQGPSPCEHGGSCINTPGSFNCLCLPGYTGSRCEADHNECLSQP
481 CHPGSTCLDLLATFHCLCPPGLEGRLCEVEVNECTSNPCLNQAACHDLLNGFQCLCLPGFTGARCEKDMDECSSTPCANG
561 GRCRDQPGAFYCECLPGFEGPHCEKEVDECLSDPCPVGASCLDLPGAFFCLCRPGFTGQLCEVPLCTPNMCQPGQQCQGQ
641 EHRAPCLCPDGSPGCVPAEDNCPCHHGHCQRSLCVCDEGWTGPECETELGGCISTPCAHGGTCHPQPSGYNCTCPAGYMG
721 LTCSEEVTACHSGPCLNGGSCSIRPEGYSCTCLPSHTGRHCQTAVDHCVSASCLNGGTCVNKPGTFFCLCATGFQGLHCE
801 EKTNPSCADSPCRNKATCQDTPRGARCLCSPGYTGSSCQTLIDLCARKPCPHTARCLQSGPSFQCLCLQGWTGALCDFPL
881 SCQKAAMSQGIEISGLCQNGGLCIDTGSSYFCRCPPGFQGKLCQDNVNPCEPNPCHHGSTCVPQPSGYVCQCAPGYEGQN
961 CSKVLDACQSQPCHNHGTCTSRPGGFHCACPPGFVGLRCEGDVDECLDRPCHPSGTAACHSLANAFYCQCLPGHTGQRCE
1041 VEMDLCQSQPCSNGGSCEITTGPPPGFTCHCPKGFEGPTCSHKALSCGIHHCHNGGLCLPSPKPGSPPLCACLSGFGGPD
1121 CLTPPAPPGCGPPSPCLHNGTCTETPGLGNPGFQCTCPPDSPGPRCQRPGASGCEGRGGDGTCDAGCSGPGGDWDGGDC
1201 SLGVPDPWKGCPPHSQCWLLFRDGRCHPQCDSEECLFDGYDCEIPPTCIPAYDQYCRDHFHNGHCEKGCNNAECGWDGGD
1281 CRPEGEDSEGRPSLALLVVLRPPALDQQLLALARVLSLTLRVGLWVRKDSEGRNMVFPYPGTRAKEELSGARDSSSWERQ
1361 APPTQPLGKETESLGAGFVVVMGVDLSRCGPEHPASRCPWDSGLLLRFLAAMAAVGALEPLLPGPLLAAHPQAGTRPPAN
1441 QLPWPILCSPVVGVLLLALGALLVLQLIRRRRREHGALWLPPGFIRRPQTQQAPHRRRPPLGEDNIGLKALKPEAEVDED
1521 GVAMCSGPEEGEAEETASASRCQLWPLNSGCGELPQAAMLTPPQECESEVLDVDTCGPDGVTPLMSAVFCGGVQSTTGAS
1601 PQRLGLGNLEPWEPLLDRGACPQAHTVGTGETPLHLAARFSRPTAARRLLEAGANPNQPDRAGRTPLHTAVAADAREVCQ
1681 LLLASRQTTVDARTEDGTTPLMLAARLAVEDLVEELIAARADVGARDKRGKTALHWAAAVNNARAARSLLQAGADKDAQD
1761 SREQTPLFLAAREGAVEVAQLLLELGAARGLRDQAGLAPGDVARQRSHWDLLTLLEGAGPTTQEARAHARTTPGGGSAPR
1841 CRTLSAGARPRGGGACLQARTWSVDLGARGGKVYARCRSRSGSCGGPTTRGRRFSAGSRGRRGARASQDDWPRDWVALEA
1921 CGSACSAPIPPPSLTPSPERGSPQVAWGLPVHQEIPLNSVVRNLN 1964
EGF- like repeats
Notch/lin-12 repeats
Transmembrane domain
cdc10 / ankyrin repeats

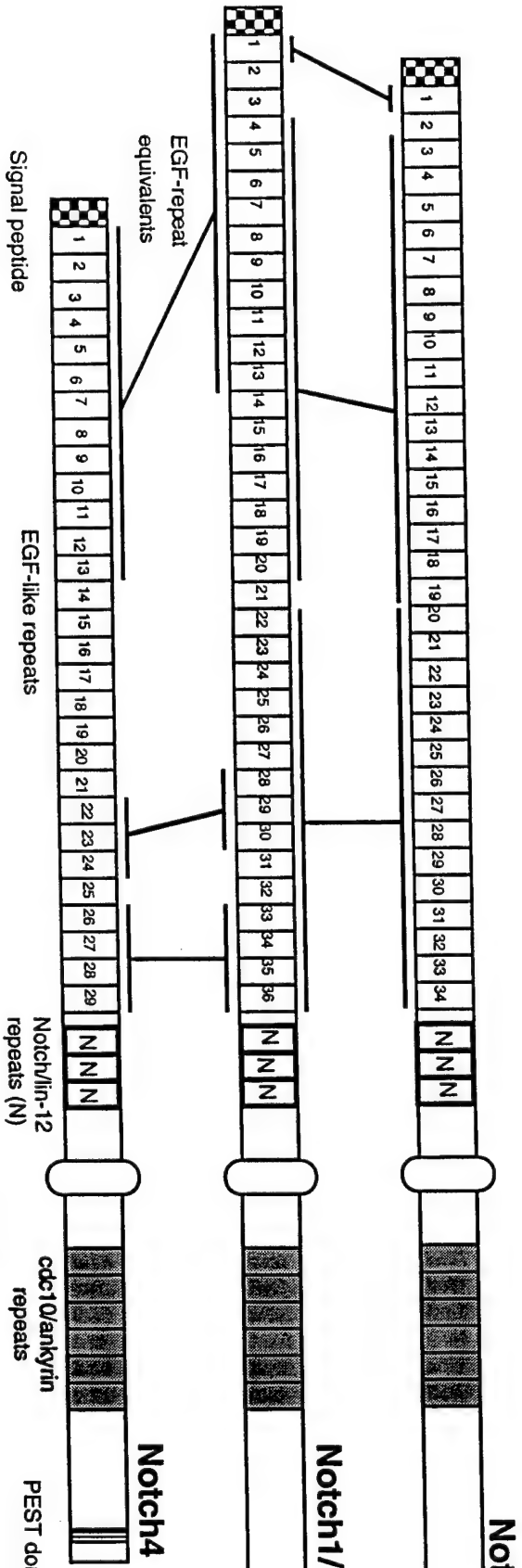
Analysis of the *Notch4* deduced amino acid sequence

Analysis of the deduced amino acid sequence of *Notch4* reveals the presence of conserved domains shared by all Notch proteins (see Fig. 1). Notch4 contains EGF-like repeats, Notch/lin-12 repeats, a transmembrane domain, cdc10/ankyrin repeats and a putative PEST domain. The overall homology between Notch4 and other Notch proteins was determined using GCG (Bestfit, gap weight 3.0, length weight 0.1). The Notch4 protein is approximately 60% similar and 43% identical to other vertebrate Notch proteins, and 58% similar and 40% identical to *Drosophila* Notch. Lower homologies were found when compared to the *C. elegans* lin-12 and glp-1 proteins (49% similar and 29% identical).

Two hydrophobic regions in the Notch4 protein sequence were identified by hydropathy analysis (Kyte Doolittle algorithm, data not shown). An N-terminal region contains 19 hydrophobic residues that could function as a signal peptide sequence (Fig. 1) and a putative signal peptidase cleavage site was identified at residue 20. A second hydrophobic region from amino acid residues 1441 to 1465 is of sufficient length (25 amino acids) to behave as a membrane spanning domain and is immediately followed by five consecutive arginine residues that are consistent with a stop transfer signal (Fig. 1).

The extracellular domain of Notch4 contains 29 EGF-like repeats (Figs. 1, 2), in contrast to the 36 EGF-like repeats found in murine Notch 1 (Franco Del Amo et al., 1993) and rat Notch 2 (Weinmaster et al., 1992), and to the 34 EGF-like repeats found in murine Notch 3 (Lardelli et al., 1994). EGF-like repeats are defined by a cysteine-rich consensus sequence and generally occur in analogous locations in two different Notch proteins. Since analogous repeats are more homologous to each other than to their neighboring EGF-like repeats, they have been referred to in Notch proteins as equivalent EGF-like repeats. We analyzed the relationship between particular EGF-like repeats of other Notch proteins and those of the Notch4 protein. Figure 2 schematizes the relationship of

Fig. 2 Schematic structural comparison of the four murine Notch proteins. The EGF-like repeats are numbered according to their position in each different protein. Where equivalent EGF-like repeats can be identified connecting lines are placed to compare the relationship between these repeats in different Notch proteins (see EGF-repeat equivalents). Notch4 contains 7 EGF-like repeats less than Notch1 and Notch2. One of the missing EGF-like repeats (#25) in Notch4 is derived from equivalent repeats #31 and #32 of Notch1/Notch2 creating a novel and hybrid EGF-like repeat. Eight of the EGF-like repeats of Notch4 (#14 to #21) have no identifiable equivalent repeats in Notch1/Notch2. The region of Notch4 from the end of the cdc10/ankyrin repeats to the carboxy-terminus is shorter when compared to Notch 1, 2, and 3.

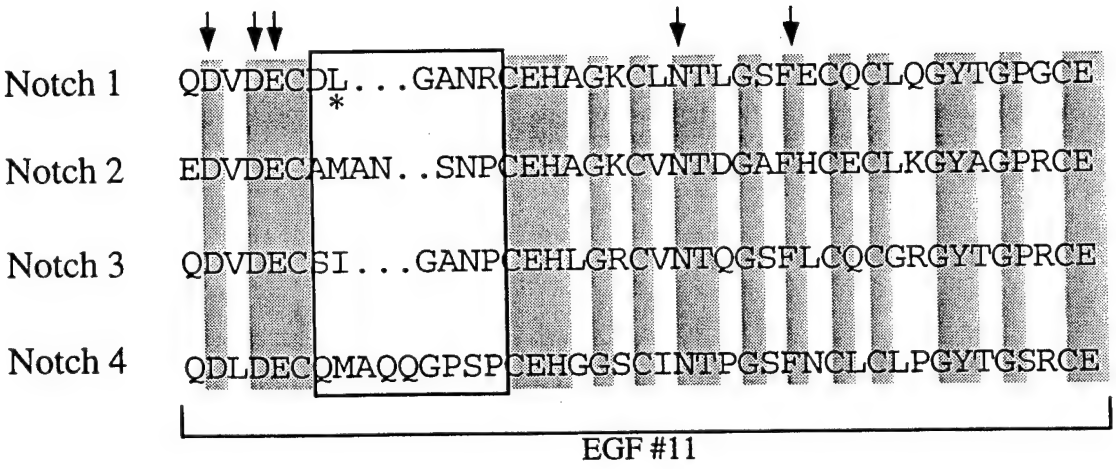

Notch3
Notch1/Notch2
Notch4
EGF-repeat equivalents
Signal peptide
EGF-like repeats
Notch/lin-12 repeats (N)
Transmembrane domain
cdc10/ankyrin repeats
PEST domain

EGF-equivalents between Notch4 and Notch1/Notch2. EGF-like repeats 1-13 of Notch4 are equivalent to EGF-like repeats 1-13 of Notch1/Notch2 , EGF-like repeats 22-24 of Notch4 correspond to EGF-like repeats 28-30 of Notch1/Notch2, and EGF-like repeats 26-29 of Notch4 are equivalent to EGF-like repeats 33-36 of Notch1/Notch2. Comparison of Notch4 to other Notch proteins revealed no clear-cut identification of the seven particular equivalent EGF-like repeats that are absent in Notch4. The amino acid sequence of equivalent EGF-like repeats has diverged between different Notch homologues and orthologues (Maine et al., 1995), sometimes resulting in loss of a clear-cut equivalent repeat consensus. Six of the unassigned EGF-like repeats of Notch4 appear to be derived from EGF-like repeats 14-27 of Notch1 and Notch2 (Fig. 2). EGF-like repeat 25 of Notch4 may be a hybrid EGF-like repeats derived from parts of EGF-like repeats 31 and 32 of Notch1/Notch2 (Fig. 2). A discussion of the relationship between Notch3 and Notch1/Notch2 (shown in Fig. 2) is described in Lardelli et al., 1994.

EGF-like repeats 11 and 12 of *Drosophila* Notch have been shown to be necessary and sufficient for Notch to bind Delta and Serrate proteins *in vitro* (Rebay et al., 1991). These two equivalent EGF-like repeats are present in Notch4 (Fig. 2). The putative calcium-binding residues (Handford et al., 1991) in EGF-like repeat 11 are also conserved in Notch4 (Fig. 3). The residues between the first and second cysteine of EGF-like repeat 11 have been shown in *Xenopus* Notch to be important in ligand binding, and are divergent between Notch proteins (Fig. 3). In this region, Notch4 has additional residues and is unique when compared to other murine Notch proteins.

Notch4 also contains three Notch/lin-12 repeats which are approximately 53% identical to the Notch/lin-12 repeats found in other murine Notch proteins. Between the Notch/lin-12 repeats and the transmembrane domain of Notch4 are 2 cysteines at positions 1388

Fig. 3 Amino acid sequence comparison of EGF-like repeat #11 of mouse Notch 1, 2, 3 and 4. Residues conserved between the mouse Notch proteins are shaded and the putative calcium binding sites are marked with arrows. A region within EGF-like repeat #11 of Notch proteins containing non-conserved and variable numbers of residues is boxed. The leucine to proline mutation in *Xenopus* Notch that obliterates binding to Delta is marked with an asterisk (*).


Notch 1
QDVDECDL...GANRCEHAGKCLNTLGSFECQCLQGYTGPGCE
Notch 2
EDVDECAMAN..SNPCEHAGKCVNTDGAFHCECLKGYAGPRCE
Notch 3
QDVDECSI...GANPCEHLGRCVNTQGSFLCQCGRGYTGPRCE
Notch 4
QDLDECQMAQQGPSPCEHGGSCINTPGSFNCLCLPGYTGSRCE
*
EGF #11

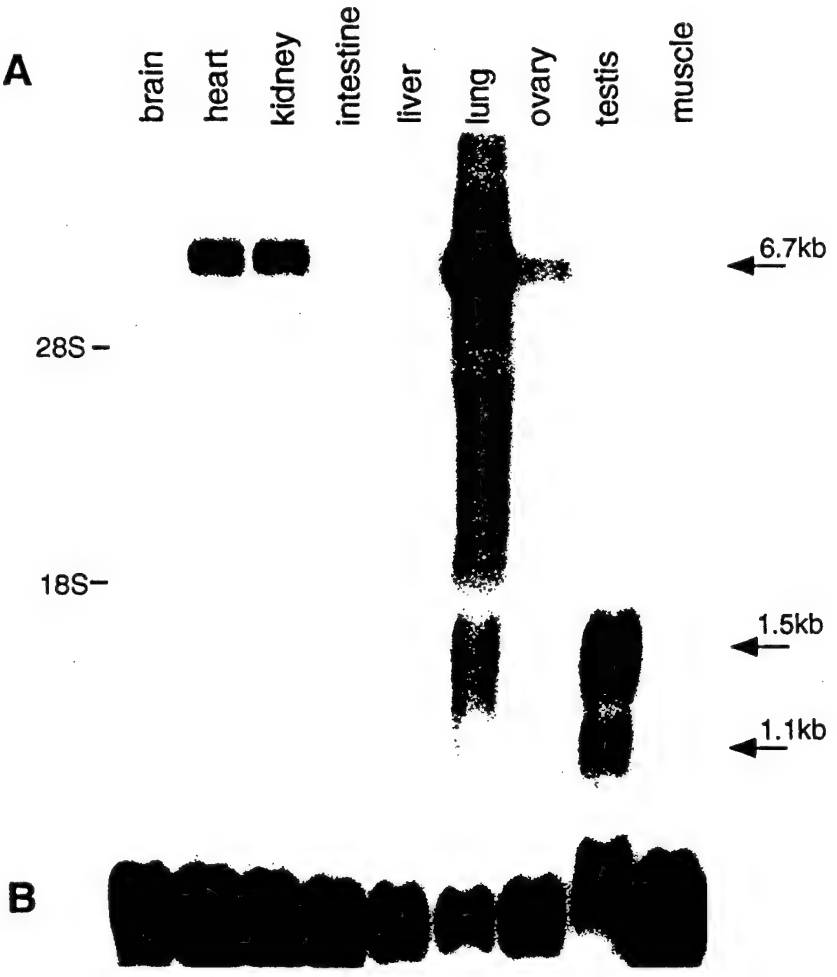
and 1397 that are conserved among all Notch proteins and may promote receptor dimerization upon ligand binding (Greenwald and Seydoux, 1990).

The intracellular domain of Notch4 contains the 6 ankyrin/cdc10 repeats found in other Notch proteins. The ankyrin repeat domain of Notch4 is 48%, 52%, and 55% identical to the ankyrin repeat domains of Notch1, Notch2, and Notch3 respectively. In all Notch proteins the number of amino acids between the transmembrane domain and the ankyrin/cdc10 repeats is 110 residues, as it is in Notch4 (Fig. 1). Like other Notch proteins, Notch4 contains a C-terminal PEST domain, albeit of shorter length. In addition, Notch4 lacks a recognizable opa repeat (Fig. 1), such as that found in *Drosophila* Notch. The carboxy-terminal end of Notch proteins, beyond the ankyrin/cdc10 repeats, is the least conserved region among Notch proteins. Within this C-terminal region, Notch4 displays little homology to other Notch proteins and no significant homology to other known proteins. This C-terminal is also much shorter in Notch4 than in other Notch proteins, containing 177 residues, compared to 457 in Notch1, 437 in Notch2, and 329 in Notch3.

Expression analysis of *Notch4* in adult tissues

Several adult tissues were examined for the presence of *Notch4* transcripts by Northern blot analysis. To minimize cross-hybridization with other mouse *Notch* transcripts, we used a riboprobe derived from the 3' UTR of *Notch4*. In most tissues analyzed, a single hybridizing species of 6.7 kb was detected (Fig. 4), which roughly corresponds in size to the cloned *Notch4* cDNA. The 6.7 kb transcript is most highly expressed in lung, at lower levels in heart and kidney, and at detectable levels in ovary and skeletal muscle. Very low levels of the 6.7 kb transcript were observed in several other adult tissues; including brain, intestine, liver, testis (Fig. 4) and spleen (data not shown). In adult testis, two abundant transcripts of 1.5 kb and 1.1 kb were observed. Thus, *Notch4*

Fig. 4 Expression analysis of Notch4 in adult mouse tissues. Panel A: Northern blot using riboprobe transcribed from the 3' UTR of *Notch4* (probe D in Fig. 5), Panel B: the same blot was reprobed with a GAPDH probe. The transcript sizes of 6.7kb, 1.5kb, and 1.1 kb are indicated and were estimated in reference to 28S and 18S rRNA migration.


A
brain
heart
kidney
intestine
liver
lung
ovary
testis
muscle
6.7kb
28S –
18S–
1.5kb
1.1kb
B

expression varies widely in adult tissues. Other than in testis, we did not detect transcript size variation in different tissues.

Analysis of testis-specific truncated *Notch4* transcripts

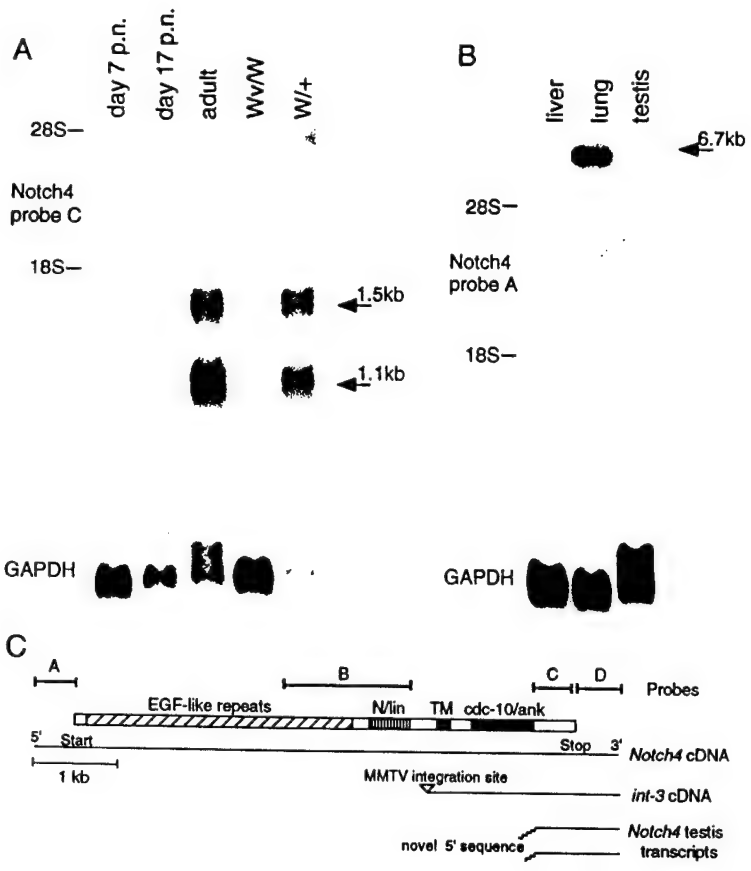
To determine the cell lineage specificity of *Notch4* expression in the murine testis, RNA was analyzed in the germ cell deficient mouse testis (Fig. 5). Mice that carry two mutations at the white-spotting locus (W/W^V) are devoid of germ cells, but have the normal complement of somatic cell types, including Leydig, Sertoli, and peritubular myoid cells (Busse and Seguin, 1993). Heterozygous litter mates (*W*/+) have normal somatic and germ cell complements. Northern blot analysis of total RNA from germ cell-deficient testes (W/W^V) and testes with normal germ cells (*W*/+ and adult (+/+)) was done using a riboprobe derived from the 3' UTR (probe D in Fig. 5C). Transcripts of 1.5 kb and 1.1 kb were detected in RNA from the testes of adult *wild-type* mice and *W*/+ mice (Fig. 5A). However, neither transcript was detected in RNA from homozygous mutant testes, suggesting that these transcripts were likely to be specific to the germinal compartment.

Since spermatogenic differentiation undergoes a characteristic temporal progression, one can use mice testes at specific days of postnatal development to enrich for or eliminate particular germ cell types. Testes from day 7 of postnatal development (day 7 p.n.) mice contain mitotic spermatogonia while testes from day 17 p.n. mice have entered meiosis and have progressed to spermatocytes (Christian et al., 1991). Both day 7 p.n. and day 17 p.n. testes lack post-meiotic spermatids. Total RNA from immature and adult testes was analyzed by Northern blot hybridization to determine stage-specific expression of *Notch4* transcripts during male germ cell development. Both *Notch4* transcripts of 1.5 kb and 1.1 kb are absent in day 7 p.n. and day 17 p.n. testis, but are present in adult testis

(Fig. 5A). These results indicate that the expression of the 1.5 kb and 1.1 kb *Notch4* transcripts are restricted to post-meiotic germ cells.

To determine the nature of the short *Notch4* transcripts in adult mouse testis, Northern blot analysis was done using riboprobes derived from different regions of the *Notch4* coding sequence, as well as from 5' and 3' UTR (Fig. 5B). A riboprobe derived from the 5' UTR (probe A in Fig. 5C) failed to hybridize to either the 1.5 kb or the 1.1 kb transcripts (Fig. 5B); whereas this probe did hybridize to the 6.7 kb transcript found in lung RNA (Fig. 5B). However, riboprobes derived from the 3' UTR (probe D in Fig. 5C) or from cDNA encoding part of the intracellular domain of Notch4 (probe C in Fig. 5C) hybridize to the testis transcripts (Fig. 5A and data not shown). Probes derived from the coding sequence of the extracellular domain of Notch4 (probe B in Fig. 5C) did not hybridize to the testes transcripts (data not shown). To characterize the transcripts expressed in the adult mouse testis, a cDNA library prepared from adult mouse testes RNA was screened using probe C of Figure 5C. All the clones analyzed encoded the most C-terminal coding sequence and the 3' untranslated region of Notch4. Two independent clones of distinct size contained novel 5' sequences unrelated to that found in the full-length *Notch4* cDNA (schematized in Fig. 5C-*Notch4* testis transcripts). Based upon the Northern blot analysis described above and the sequence of the cloned testis cDNAs, we believe that *Notch4* transcripts are either derived from an alternate intronic promoter that is active in post-meiotic germ cells or they may be driven by the same promoter as the 6.7 kb transcript and consist of spliced products derived from a 5' untranslated region upstream of what we have currently identified. The predicted amino acid sequence of the testis *Notch4* transcripts with novel 5' sequence does not contain a methionine that could function as a translation initiator; therefore, these transcripts are unlikely to encode for protein products. The testis transcripts may thus represent aberrant

Fig. 5 Expression analysis of Notch4 testis transcripts. Panel A: Notch4 testis transcripts are expressed in post-meiotic germ cells. Northern blot analysis from staged and germ cell deficient testes with probe C and a GAPDH probe. Note that GAPDH transcripts appear as two isoforms in the adult testis. RNA was isolated from testes of day 7 p.n., day 17 p.n., adult, $W^v/W_,$, and *W/+* mice as indicated. Panel B: Northern blot analysis of several adult tissues with probe A, derived from the 5' UTR of Notch4, and a GAPDH probe. Panel C: Schematic representation of truncated Notch4 transcripts as compared to the full length coding potential. Relative position of probes used in Northern blot analysis is shown. Conserved elements of Notch family proteins are indicated. MMTV integration site reported in (Robbins et al., 1992) is indicated by an arrow. Novel 5' sequences of testes cDNAs are indicated.


A
day 7 p.n.
day 17 p.n.
adult
Wv/Wv
W/+
28S—
Notch4
probe C
18S—
1.5kb
1.1kb
GAPDH
B
liver
lung
testis
6.7kb
28S—
Notch4
probe A
18S—
GAPDH
C
A
B
C
D
Probes
EGF-like repeats
N/lin
TM
cdc-10/ank
5'
Start
Stop
3'
Notch4 cDNA
1 kb
MMTV integration site
int-3 cDNA
Notch4 testis
transcripts
novel 5' sequence

transcriptional events in post-meiotic germ cells, as has been described previously (Davies and Willison, 1993).

Expression analysis of *Notch4* during development and in adult lung

A 6.7 kb *Notch4* transcript was detected by Northern hybridization in RNA isolated from day 12.5 p.c. mouse embryos (data not shown and (Sarkar et al., 1994)Sarkar et al., 1994) and adult lung (Fig. 4). To determine the spatial and temporal pattern of *Notch4* transcript accumulation during development, we examined mouse embryo tissue sections from 9.0 days p.c. to birth using in situ hybridization. During embryonic development, as well as in postnatal tissues, *Notch4* is highly expressed in endothelial cells. Intense labeling for *Notch4* is observed in embryonic blood vessels at 9.0 days p.c. (Fig. 6A, B). As shown in Fig. 6 (panel C and D) strong labeling is observed over the dorsal aorta, the aortic tract, and the pulmonary artery in a 13.5 days p.c. embryo, while no labeling is detected in the epithelial cells lining the gut (red arrow). At higher magnification, we note that labeling is restricted to the endothelial cells lining the embryonic vessels (Fig. 6 D, E) and no labeling is detected in the red blood cells in the vessel. A weak and transient signal is also detectable in the developing nervous system from 9.0 days p.c. embryos. As shown in Figure 6 (panel A and B), a light diffuse labeling is detected in the developing nervous system, and a more distinct signal is observed at the tip of the neural folds. *Notch4* transcripts in the nervous system are still detectable at 11.5 days p.c., but by 13.5 days p.c. no labeling for *Notch4* is detectable in the nervous system (data not shown).

Since adult lung exhibited the highest levels of *Notch4* transcripts, in situ hybridization was performed on lung sections to determine whether *Notch4* expression remains endothelial cell specific in adult life. Intense punctate staining was observed over the alveolar wall indicative of capillary specific expression (Fig. 7). The central component

of the alveolar wall is the capillary flanked by pneumocyte type I epithelial cells which line the alveolar lumen (Ross and Reith, 1985). Capillaries are highly localized in the alveolar wall and would give the punctate localized signal observed, as opposed to a more uniform pattern for epithelial cells lining the alveolar cavity. There is clearly no hybridization signal over other cellular components of the lung; that is, pseudostratified squamous epithelium, smooth muscle, and connective tissue cells. The endothelial specific expression likely underlies the abundance of *Notch4* transcripts found by Northern blot analysis of highly vascularized adult tissues (lung, heart, and kidney in Fig. 4).

Fig. 6 Notch4 is expressed in embryonic endothelial cells. Panel A and B: phase and darkfield photomicrograph of a horizontal section of a 9 days p.c. embryo hybridized with a cRNA probe corresponding to Notch4. Strong labeling is detectable over the anterior cardinal vein (white arrow). Diffuse labeling is also present throughout the developing nervous system and at higher level over the tip of the neural folds (red arrow). Panel C-F: Phase and darkfields images of a horizontal section of a 13.5 days p.c. embryo hybridized for Notch4 showing the venous and arterial system anterior to the lung, including dorsal aorta arch, aortic and pulmonary tract. Panel E and F are higher magnification of the area framed in panel C. Embryonic vessels are labeled and, as shown in panel E and F, labeling is restricted to the endothelial cells lining the vessels. Arrow denotes the gut which does not have detectable signal in the epithelium.

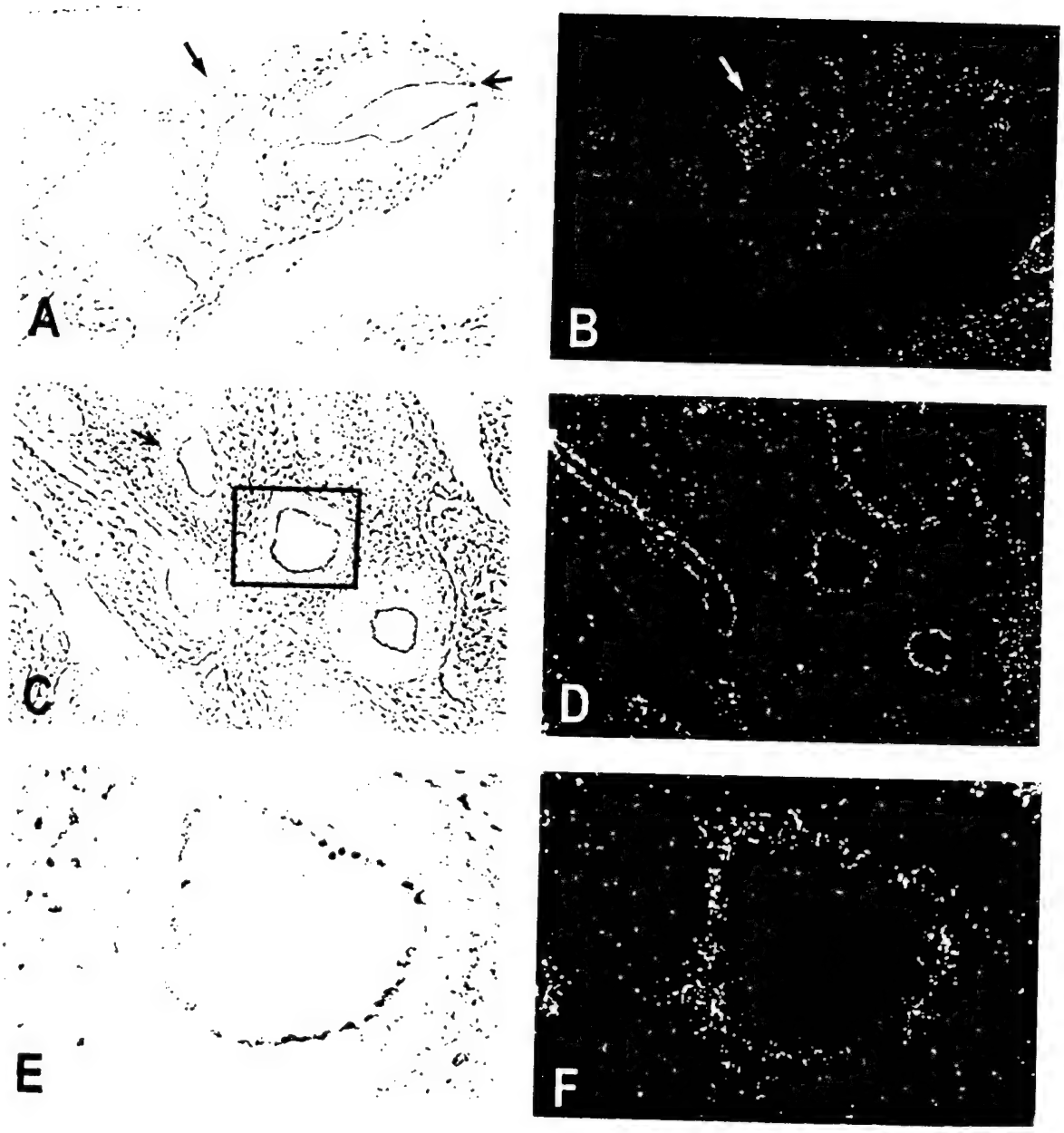
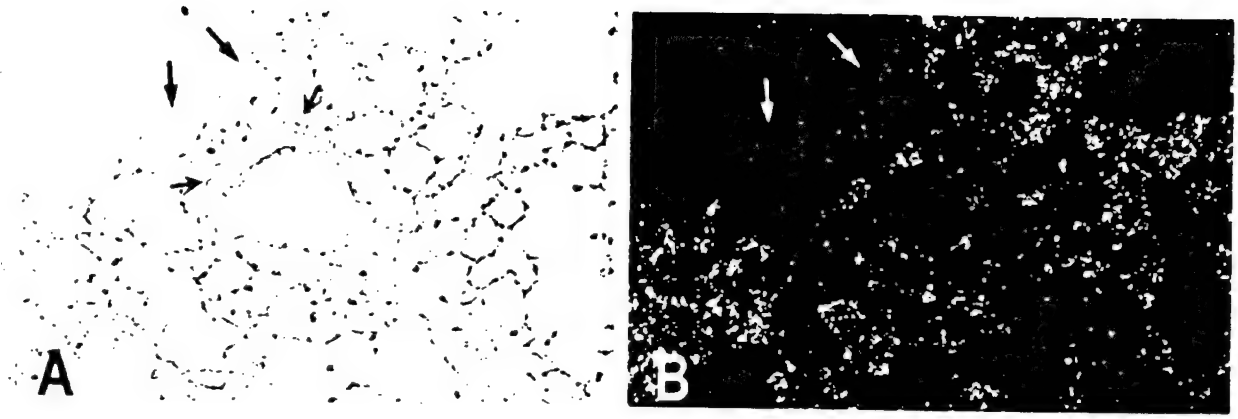

A
B
C
D
E
F

Fig. 7 Notch4 is expressed in adult lung endothelial cells. Panel A and B: phase and darkfield photomicrographs of an adult mouse lung hybridized with a cRNA probe corresponding to Notch4. Punctate staining is observed over the alveolar walls in a pattern indicative of capillaries. No labeling is observed over the pseudostratified squamous epithelium (black and white arrows) nor over the smooth muscle cells (red arrows).


A
B

Discussion

We report here the identification of a novel mouse gene that exhibits structural homology with the vertebrate Notch protein family. We have named this gene *Notch4*, as it is the fourth murine *Notch* gene identified. Notch4 contains all the conserved domains characteristic of Notch proteins (Figs 1 and 2). However, Notch4 contains only 29 EGF-like repeats within its extracellular domain as compared to the 36 repeats found in Notch1 and Notch2. In addition, the C-terminal tail of Notch4, beyond the ankyrin/cdc10 repeats, is shorter and unique when compared to all other Notch proteins, however, little is known of the function of this region in Notch proteins. Notch4 also contains a distinct EGF-like repeat 11 which has been proposed to be crucial for ligand binding. Structural variation in this repeat, and differences in the number of EGF-like repeats between murine Notch proteins, may be important for ligand specificity among the different possible Notch ligands. It must be noted that Notch/lin-12 proteins of varying structure have been demonstrated to be functionally interchangeable; *C. elegans* glp-1 can fully substitute for lin-12 (Fitzgerald et al., 1993) for instance. Therefore, Notch4 may be functionally interchangeable with other murine Notch proteins despite structural differences between these Notch proteins.

Notch4 is distinct from other Notch family proteins based on its expression pattern during embryonic development and in the adult mouse. In situ hybridization demonstrates endothelial-specific embryonic expression of *Notch4*. This endothelial-specific expression of *Notch4* remains in the adult mouse. A weak and transient labeling is seen in the neural tube between day 9 p.c. and 11.5 p.c., with a more intense labeling at the tips of neural folds. This region of the neural tube is a highly plastic area where cells will probably participate in the fusion process of the neural tube and/or migrate as neural crest. The *Notch4* expression pattern is in sharp contrast to the expression patterns of

Notch1, *2*, and *3*. These *Notch* genes are expressed in a variety of different embryonic tissues such as the developing brain and spinal cord, presomitic and somitic mesoderm, and a variety of epithelial cells and mesenchymal derived tissues (Weinmaster et al., 1991; Williams et al., 1995). *Notch1* is the only other *Notch* gene reported to be expressed in endothelial cells (Reaume et al., 1992). (Bettenhausen et al., 1995; Lindsell et al., 1995). Expression of Notch1 and 4 in endothelial cells might reflect either redundancy of function or distinct biological functions in endothelial development. Endothelial cell specific expression has recently been reported for a putative Notch ligand, the chick *Serrate* homologue (Myat et al., 1996).

Since Notch proteins have been implicated in binary cell fate specification regulating how equivalent cells can give rise to cells with different fates, a putative biological function of Notch4 might be to govern the cell fate decisions during endothelial growth and development. In amniotes, endothelial and hematopoietic cells appear synchronously in the blood islands. In zebra fish, lineage data have shown that individual cells of the early blastula can give rise to both endothelial and blood cells, suggesting a common embryonic precursor which has been referred to as the "hemangioblast." The occurrence of binary cell fate decision events in the hemangioblast is supported by analysis of the endothelial and/or hematopoietic cell lineages. *Cloche*, *bloodless*, and *spadetail* are mutants isolated in zebra fish that display phenotypes defective in either the hematopoietic development or both hematopoietic and endothelial development (Stainier et al., 1995). In the mouse, the *Flk-1* and the *Flt-1* genes encode receptor tyrosine kinases that are expressed in embryonic endothelium (Shalaby et al., 1995, Fong et al., 1995). Null mutants for the *Flk-1* gene are defective in endothelial and blood cell development (Shalaby et al., 1995), whereas null mutants for the *Flt-1* gene display only hematopoietic cell development defects (Fong et al., 1995). Mutational analysis of the *Notch4* gene in

whole animals would help to define the role of Notch4 in endothelial cell growth and development.

Alterations in stem cell fate decisions as a result of activated Notch proteins have been proposed to contribute to mitogenic growth of tumor cells. Blocked cell differentiation of fated daughter cells by activated Notch proteins may lead to an increase in the number of cells undergoing cell division or a prolonged life of the cell. In these cells, the probability of secondary oncogenic mutations that contribute to neoplastic transformation would be enhanced. In the normal mouse mammary gland, endogenous int-3 protein has been detected at low levels in mammary stroma and epithelium (Smith et al., 1995). Although little is known about the nature of stem cells in the mammary epithelium, Notch4 might regulate the fate decisions of mammary epithelial cells. This hypothetical model may explain the phenotype that is observed in *int-3* transgenic mice, which display blocked development of the mammary gland and develop mammary carcinomas at high frequency.

The signal transduction pathways by which Notch proteins function are becoming understood through genetic studies in *Drosophila*. Deltex and Suppresser of Hairless (Su(H)) have been demonstrated to bind to the cdc10 repeats of the intracellular domain of *Drosophila* Notch (Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Matsuno et al., 1995). More recently the mammalian Su(H) orthologue RBP-Jk, a transcription factor, has been shown to bind to the intracellular domain of Notch 1 (Jarriault et al., 1995). Since Notch4 contains the canonical ankyrin/cdc10 repeats, RBP-Jk or RBP-Jk homologues and mammalian Deltex homologues may interact with the cdc10/ankyrin repeats of Notch4. It has been proposed that upon activation of the Notch receptors, Su(H) or RBP-Jk are activated and translocate to the nucleus where they may regulate transcription of target genes (Goodbourn, 1995). In fact, activated Notch

proteins encoding the intracellular domain have been reported to localize to the nucleus (Kopan et al., 1994; Struhl et al., 1993) suggesting a nuclear function for this domain. We have found that the int-3 oncoprotein, modified to encode a flu epitope-tag at the C-terminus, is also localized to the nucleus when expressed in cultured 293T cells, as determined by immunofluorescence (unpublished data). The activated int-3 protein lacks a signal peptide but contains a membrane spanning domain and thus is not likely to enter the secretory pathway. This finding may indicate that int-3 can bind to cytoplasmic proteins that are then translocated to the nucleus.

We show that the int-3 gene encodes a truncated Notch4 protein with the extracellular domain deleted (EGF-like repeats and Notch/lin-12 repeats), providing the first comparison of a naturally-activated murine Notch protein and its normal counterpart. In MMTV-induced mouse mammary tumors with an activated Notch4, as described in Robbins et al. (1992), the oncogenic affects are likely the result of both overexpression or ectopic expression of *Notch4* mRNA as well as functional activation of the Notch4 protein. A structural comparison of the mutant int-3 protein to the normal Notch4 protein is reminiscent of the structural alterations reported to activate the effector function of *Drosophila* Notch and *C. elegans* lin-12 proteins (Greenwald, 1994) or oncogenic activation of TAN-1. Thus, loss of the extracellular domain likely leads loss of the regulatory controls provided by the ligand binding domain believed to reside in the EGF-like repeats of Notch4.

Chapter 4

Notch and Wnt proteins function to regulate branching morphogenesis of mammary epithelial cells in opposing fashion

Introduction

The development of the murine mammary gland involves an intricate sequence of proliferative, morphogenetic and differentiative events, which gradually results in the formation of an arborized tree-like structure of epithelial ducts. Postnatal development of the mammary gland is influenced by gonadal hormones, with distinct developmental stages occurring during puberty, estrous, pregnancy and lactation. At birth, the mammary epithelial ducts have few side-branches. During puberty, the epithelial ducts rapidly elongate and branch, and give rise to a highly organized epithelial structure with terminal end buds and lateral buds. The terminal end buds are the major sites of proliferation, whereas the lateral buds differentiate into alveoli during each estrous cycle. During pregnancy, the alveoli rapidly increase in size and number resulting in the development of fully differentiated lobules, which will produce milk at lactation. The mammary gland remodels after lactation ceases, and this process is characterized by the involution of the secretory lobules and regression to the ductal tree observed at puberty (review in Daniel and Silberstein, 1987; Pitelka *et al.*, 1973; Russo *et al.*, 1989).

Mesenchymal-epithelial and epithelial-epithelial interactions are essential in the regulation of growth and development of the murine mammary gland. Peptide growth factors, such as Epidermal growth factor (EGF) (Haslam et al., 1992), Fibroblast growth factors (FGF) (Coleman-Krnacik and Rosen, 1994), Hepatocyte growth factor (HGF), Insulin-like growth factor II (IGF-II) (Bates et al., 1995), Neuregulin (NRG) (Yang et al., 1995), and Transforming growth factor-β (TGF-β) (Daniel et al., 1996; Pierce et al., 1993), have been implicated as regulators of mammary gland development based on their expression patterns and, in some cases, on their abilities to affect the development of the mammary gland. HGF (or scatter factor) is expressed in the mammary mesenchyme during ductal branching, whereas its tyrosine kinase receptor c-met is expressed in the

mammary epithelial ducts at all stages (Niranjan et al., 1995). HGF can promote branching morphogenesis of the mammary ductal tree (Niranjan et al., 1995; Pepper et al., 1995; Soriano et al., 1995; Yang et al., 1995) in several experimental settings. TGF-β1 is expressed in the epithelial compartment of the mammary gland at all stages, except during lactation (Daniel et al., 1996; Smith, 1996). *In vivo*, TGF-b1 has been shown to inhibit ductal out-growth from the mammary end buds (Kordon et al., 1995; Pierce et al., 1993). *In vitro* however, TGF-β1 has been shown to induce opposite effects depending on its concentration. TGF-β1 at high concentrations (0.5-5 ng/ml) inhibit ductal elongation and branching of TAC-2 mammary epithelial cells, whereas at low concentrations (5-100 pg/ml) it is able to stimulate these biological processes (Soriano et al., 1996).

The Wnt family of secreted growth factors are also implicated as regulators of the developing mouse mammary gland (Nusse and Varmus, 1992). *Wnt* genes are expressed during ductal development of the gland (*Wnt*-2, *Wnt*-5a, *Wnt*-7 and *Wnt*-10b) and during lobular development at pregnancy (*Wnt*-4, *Wnt*-5b and *Wnt*-6), and the expression of most *Wnt* transcripts is down regulated during lactation (Gavin and McMahon, 1992; Weber-Hall et al., 1994). This pattern of expression during periods of morphogenesis has led to a proposed role for *Wnt* genes in morphogenetic events during mammary gland development. *Wnt* gene expression has been documented in both the stromal and epithelial compartments of the mammary gland, raising the possibility of involvement in both stromal-epithelial and epithelial-epithelial interactions (Buhler et al., 1993; Weber-Hall et al., 1994). The *Wnt*-1 gene is not normally expressed within the mouse mammary gland, however its expression can contribute to tumorigenesis when activated by insertion of mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary tumors (Nusse et al., 1984). Mammary gland tumors develop in transgenic mice where ectopic *Wnt*-1 gene expression is controlled by the MMTV promoter; these mice display

hyperplasia of the mammary epithelium and an increased incidence of tumors (Tsukamoto et al., 1988).

The *Notch4* gene was also identified as a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (Robbins et al., 1992; Sarkar et al., 1994). The *Notch4* gene encodes for a large transmembrane receptor protein (Gallahan and Callahan, 1997; Uyttendaele et al., 1996). The int-3 oncoprotein is activated by MMTV insertion and corresponds to a truncated form of Notch4 which has most of its extracellular domain deleted (Uyttendaele et al., 1996); this mutated version of Notch4 will be referred to as Notch4/int-3. In contrast to *Wnt*-1, expression of the *Notch4/int-3* oncogene as a transgene in the mouse mammary gland results in impaired development of the mammary gland which no longer generates a tree-like structure of epithelial ducts. Instead, a hyperproliferative mass of undifferentiated epithelial cells is observed near the nipple, from which undifferentiated mammary carcinomas rapidly develop (Jhappan et al., 1992).

The aim of this study was to define the roles of both Wnt and Notch signaling in mammary gland ductal morphogenesis. Using a previously described model in which TAC-2 mammary epithelial cells grown in collagen gels form branching cords or tubules in response to HGF or TGF-β1 (Soriano et al., 1996; Soriano et al., 1995), we demonstrate that activation of the Wnt and Notch signaling pathways has opposite effects on branching morphogenesis. Wnt-1 acts to induce branching morphogenesis whereas Notch4/int-3 inhibits branching morphogenesis by either HGF or TGF-β. Wnt-1 has the capacity to overcome the Notch4/int-3 mediated inhibition of branching morphogenesis.

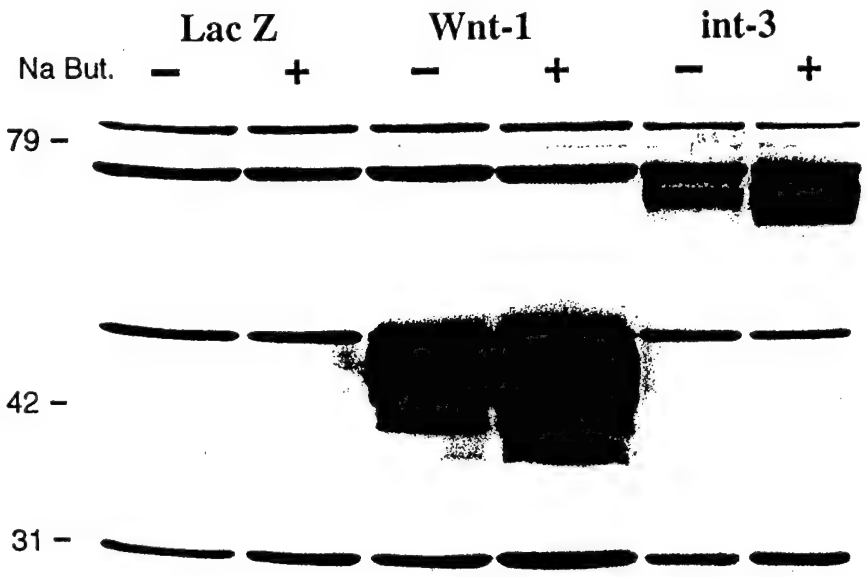
Results

When suspended in collagen gels, TAC-2 mammary epithelial cells form small slowly growing colonies with a morphology ranging from irregular shaped cell aggregates to poorly branched structures. Under these same conditions, TAC-2 cells grown in the presence of either HGF or TGF-β1 develop an extensive network of branching cords that consist of elongated epithelial cords or tubules with multiple branch points (Soriano et al., 1996; Soriano et al., 1995). This TAC-2 cell phenotype is thus reminiscent of the branching morphogenesis of epithelial ducts in the mammary gland and provides an experimental model to study the roles of growth factors and receptors in the development of the mammary gland. To investigate the role of Wnt and Notch signaling in mammary epithelial cell growth and morphogenesis we ectopically expressed either the Wnt-1 or activated Notch4/int-3 oncoproteins and analyzed their effects on branching morphogenesis of TAC-2 cells. The results described below represent those found with several independently derived cell lines, including independent lines that were programmed to express proteins using a different promoter, as noted in the text.

Wnt-1 stimulates TAC-2 cell branching morphogenesis.

The biological activity of Wnt proteins was evaluated by generating TAC-2 cells ectopically expressing a *Wnt*-1 cDNA. TAC-2 cell lines programmed to express *Wnt*-1 (TAC-2 Wnt-1) were generated using the retroviral vector pLNCX to drive *Wnt*-1 expression from the CMV promoter . As a control, TAC-2 cells were generated that were programmed to express LacZ (TAC-2 LacZ). To evaluate the expression levels of Wnt-1 proteins in the cell lines generated, the *Wnt*-1 cDNA was fused at the carboxy terminus to the haemagglutinin-epitope (HA) tag, allowing us to detect Wnt-1 proteins in immunoblot analysis using the anti-HA monoclonal antibody (Fig. 1). Cell extracts from TAC-2 cell lines contained Wnt-1 proteins (Fig. 1) that migrated as a series of proteins

Fig. 1 Immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies. TAC-2 cells programmed to express either LacZ, Wnt-1 or int-3 were grown in the presence or absence of sodium butyrate. Wnt-1 and int-3 proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by sodium butyrate treatment.


Lac Z
Wnt-1
int-3
Na But.
– + – + – +
79 –
42 –
31 –

with molecular weights between 41 and 45 kD, due to differential glycosylation. The ectopic expression of Wnt-1 proteins in TAC-2 Wnt-1 cells can be significantly increased by treating cells with sodium butyrate (2mM), which enhances transcription of the CMV promoter (Fig. 1). In order to evaluate the effects of different protein levels on branching morphogenesis of TAC-2 cells, experiments were done either in the presence or absence of sodium butyrate. We found that addition of sodium butyrate to the TAC-2 branching morphogenesis assay did not alter or enhance the TAC-2 cell phenotypes described below.

TAC-2 cells programmed to express LacZ give rise to small colonies with poorly branched cords when grown in collagen gels for four days (Fig. 2A). Addition of either HGF (20 ng/ml) or TGF-β2 (50 pg/ml) to the culture induces pronounced changes in colony morphology, resulting in the formation of long branching cords or tubules (Fig. 2B and 2C). We utilized TGF-β2 in our assays, which we found has an identical activity as TGF-β1 in the induction of branching morphogenesis of TAC-2 cells (Soriano et al., 1996). When TAC-2 cells are programmed to express Wnt-1 proteins, cell colonies form cords with moderate branching even in the absence of exogenous growth factors (compare Fig. 2A and 2D). When TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGF-β2 (Fig. 2E and 2F), a highly extensive branching network is observed. This network of epithelial tubules is significantly more extensive when compared to control TAC-2 LacZ cells grown under identical conditions. An identical phenotype was observed in TAC-2 cell lines programmed to express either a non-epitope tagged *Wnt*-1 cDNA or a HA epitope tagged *Wnt*-1 cDNA transcribed from a SV40 based retroviral vector (data not shown). Thus, Wnt-1 activity was confirmed in at least three independently produced TAC-2 cell lines.

Fig. 2 TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ (A,B,C), Wnt-1HA (D,E,F), or int-3HA (G,H,I) were grown in collagen gels either in the absence of exogenous growth factor (A,D,G), in the presence of HGF (B,E,H), or TGF-β2 (C,F,I). HGF and TGF-β2 induce branching morphogenesis of TAC-2 LacZ cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 cells in the absence of either HGF or TGF-β2 (compare D to A), and robust branching is observed when TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGF-β2 (compare E to B, and to C). TAC-2 cells programmed to express int-3 fail to undergo branching morphogenesis when grown in the presence of either HGF or TGF-β2 (compare G to H or I, H to B, and I to C).

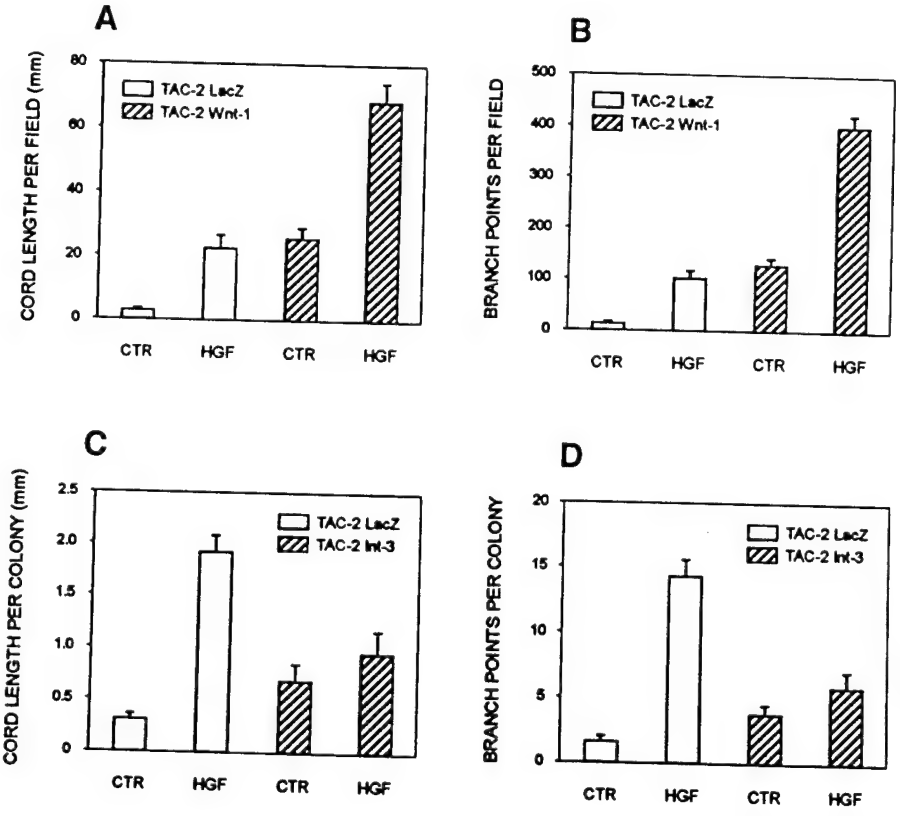
–
+ HGF
+ TGFβ
A
B
C
D
E
F
G
H
I

The morphological analysis of TAC-2 cell cultures suggested that Wnt-1 cooperates with either HGF or TGF-β2 in the induction of branching morphogenesis. To characterize combined effects of Wnt-1 and HGF, quantitative evaluation of cord length and the number of branch points was conducted (Fig. 3A and 3B). Both analyses showed that the branched network formed by TAC-2 Wnt-1 cells grown under control conditions was comparable to that found for HGF-treated TAC-2 LacZ cultures. When TAC-2 Wnt-1 cells are grown in the presence of HGF, both cord length and number of branch points is significantly greater than the combined values for TAC-2 Wnt-1 cells without HGF and TAC-2 LacZ cells grown with HGF (Fig. 3A and 3B). Thus, Wnt-1 and HGF act in a cooperative fashion to induce branching morphogenesis of TAC-2 cells.

To determine whether the effects of Wnt-1 on TAC-2 cell branching morphogenesis are due to effects on the growth characteristics of TAC-2 cells we compared the growth of the TAC-2 cell lines generated. TAC-2 cells were plated at different densities on collagen coated dishes, either in the presence or absence of HGF, and viable cell numbers were determined either two or six days after plating. No significant differences in cell number were found between control TAC-2, TAC-2 LacZ, or TAC-2 Wnt-1 cell lines grown in either the presence or absence of HGF (data not shown). When grown under these conditions, TAC-2 Wnt-1 cells and control TAC-2 cells both displayed contact inhibition at confluence and had similar morphological characteristics. Hence, the effects of Wnt-1 on TAC-2 branching morphogenesis are not correlated with mitogenic activity of Wnt-1 and are dependent on growth in three dimensional collagen gels. Identical results were obtained with TAC-2 cell lines that were programmed to express Wnt-1 using a SV40 based retroviral vector.

To further characterize morphogenetic activities of Wnt-1 proteins, we analyzed TAC-2 cells induced to form cyst structures in collagen gel cultures. When TAC-2 LacZ cells

Fig. 3 Wnt-1 and HGF have cooperative effects on branching morphogenesis of TAC-2 cells, while expression of int-3 inhibits HGF-induced branching morphogenesis. TAC-2 LacZ, TAC-2 Wnt-1 and TAC-2 int-3 cells were suspended in collagen gels at $5x10^3$ cells/ml (A and B) or $1x10^4$ cells/ml (C and D) and incubated with either control medium (CTR) or 10ng/ml HGF for 7 days. In each of 3 separate experiments, at least three randomly selected fields per condition were photographed. The total additive length of all cords in each field (A), the number of cord branch points per field (B), the total additive length of all cords in each individual colony (C), and the number of cord branch points per colony (D) was determined as described in Materials and Methods. Values are mean ± s.e.m.; n=3. Values for HGF are significantly ($P<0.001$) different when compared to controls (except for TAC-2 int-3 cells) and values are significantly different ($P<0.001$) when TAC-2 LacZ and TAC-2 Wnt-1 cell lines are compared. Similar results were obtained by evaluating cord length and branching per individual TAC-2 LacZ andTAC-2 Wnt-1 colony (data not shown).


A
CORD LENGTH PER FIELD (mm)
80
60
40
20
0
TAC-2 LacZ
TAC-2 Wnt-1
CTR
HGF
CTR
HGF
B
BRANCH POINTS PER FIELD
500
400
300
200
100
0
TAC-2 LacZ
TAC-2 Wnt-1
CTR
HGF
CTR
HGF
C
CORD LENGTH PER COLONY (mm)
2.5
2.0
1.5
1.0
0.5
0
TAC-2 LacZ
TAC-2 Int-3
CTR
HGF
CTR
HGF
D
BRANCH POINTS PER COLONY
20
15
10
5
0
TAC-2 LacZ
TAC-2 Int-3
CTR
HGF
CTR
HGF

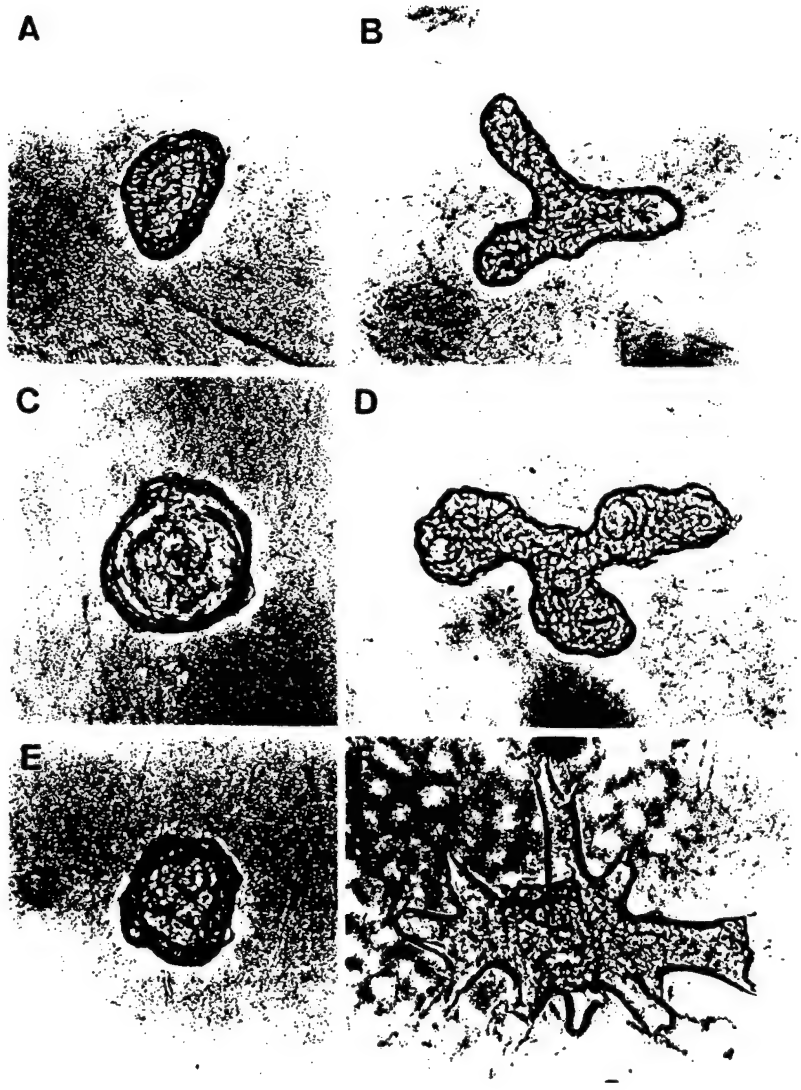
are grown in collagen gels in the presence of hydrocortisone and cholera toxin, they form spheroidal cysts enclosing a widely patent lumen, as previously observed with non-transfected TAC-2 cells (Soriano et al., 1995)(Fig. 4A). In contrast, under the same experimental conditions, TAC-2 Wnt-1 cells form branching structures (Fig. 4B). This clearly indicates that Wnt-1 expression modifies the spatial arrangement of TAC-2 cells and therefore has a morphogenetic effect.

Notch4/int-3 inhibits TAC-2 cell branching morphogenesis.

Notch4 activity in branching morphogenesis was evaluated by expressing an activated form of the Notch4 receptor, Notch4/int-3, in TAC-2 cells. TAC-2 cell lines programmed to express the Notch4/int-3 proteins were generated using the retroviral vector pLNCX and will be referred to as TAC-2 int-3. Notch4/int-3 was HA-epitope tagged at the carboxy terminus to allow detection of ectopically expressed proteins. Immunoblot analysis using anti-HA antibodies detected Notch4/int-3 proteins that migrate with an approximately molecular weight of 60 kD, corresponding well to their predicted molecular weight (Fig. 1).

When TAC-2 int-3 cells are grown in collagen gels and incubated in the presence of either HGF or TGF-β2 (Fig. 2H and 2I), cell colonies no longer form elongated cords like control cultures (Fig. 2B and 2C). Instead, HGF- or TGF-β2-treated TAC-2 int-3 cell colonies form small aggregates or structures with rudimentary branches which are similar in appearance to those formed by either TAC-2 LacZ or TAC-2 int-3 colonies grown in the absence of HGF or TGF-β2 (Fig. 2A and 2G). An identical phenotype was observed in at least three independently produced cell lines, including TAC-2 cells programmed to express a non-epitope tagged *Notch4/int-3* cDNA or a HA epitope tagged *Notch4/int-3* cDNA transcribed from a SV40 based retroviral vector (data not shown). Interestingly, we found that a smaller percentage of TAC-2 int-3 cells give rise to colonies in collagen

Fig. 4 Differential behavior of TAC-2 LacZ cells and TAC-2 Wnt-1 cells in hydrocortisone-supplemented cultures. Cells were suspended in collagen gels at $5x10^3$ cells/ml and incubated for 10 days with 1 μg/ml hydrocortisone and 50 ng/ml cholera toxin. Under these conditions, TAC-2 LacZ cells forn thick-walled spheroidal cysts enclosing a widely patent lumen (A,C,E), as previosly shown for untransfected cells. In marked contrast, TAC-2 Wnt-1 cells form branched structures consisting of either short tubules (B), cords containing small mutifocal lumina (D) or apparently solid cords (F). The three-dimensional structures illustrated in A,C,E and B,D,F are representative of the vast majority of colonies formed by TAC-2 LacZ and TAC-2 Wnt-1 cells, respectively. Magnification=180x.


A
B
C
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E

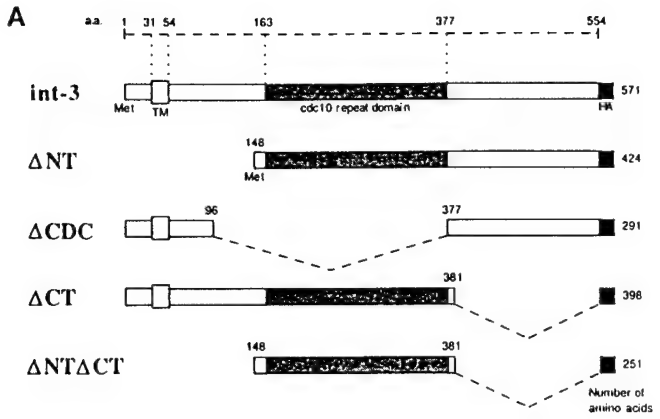
gels with respect to TAC-2 LacZ cells (230±32 colonies/cm2 in TAC-2 int-3 cells versus 795±114 colonies/cm2 in TAC-2 LacZ cells), which suggests that Notch4/int-3 expression reduces plating (colony formation) efficiency in collagen gels. Accordingly, to avoid overestimating the inhibition of HGF-induced cord elongation in TAC-2 int-3 cells, the quantitative analysis of cord length and branching was carried out on a per colony basis, rather than on a per field basis (see Materials and Methods). This analysis demonstrated that, despite the fact that colonies formed by TAC-2 int-3 cells are slightly more elongated and branched than those formed by TAC-2 LacZ cells, their morphogenetic response to HGF is markedly decreased (Fig. 3C and 3D).

We analyzed the growth characteristics of the TAC-2 int-3 cell line, as described for TAC-2 Wnt-1 cells. TAC-2 int-3 cells plated on collagen coated dishes, either in the presence or absence of HGF, displayed no significant differences in cell number, morphology, or growth post-confluence when compared with TAC-2 controls (data not shown). Identical results were obtained with TAC-2 cell lines programmed to express Notch4/int-3 using a SV40 based retroviral vector. Hence, the effects of Notch4/int-3 on TAC-2 branching morphogenesis are not correlated to changes in the growth properties in the cells.

The carboxy terminus of the Notch4/int-3 is not required for activity.

The Notch4/int-3 oncoprotein has most of the extracellular domain of Notch4 deleted and consists of the transmembrane and intracellular domains. To investigate which region(s) of Notch4/int-3 proteins are required and sufficient for activity, a series of Notch4/int-3 deletion mutants were generated (schematized in Fig. 5A). Four Notch4/int-3 deletion mutants were made and designated ΔNT (deletion of the amino terminal domain), ΔCDC (deletion of cdc10 repeat domain), ΔCT (deletion of the carboxy terminal domain) and ΔNTΔCT (N-terminal and C-terminal deletion) (Fig. 5A). All four

Fig. 5 Schematic representation of int-3 deletion mutants (A) and immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies (B,C). TAC-2 cells programmed to express either DNT, DCDC, DCT, and DNTΔCT were grown in the presence or absence of sodium butyrate. The int-3 deletion proteins are epitope tagged and deletion proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by sodium butyrate treatment. Immunoblot analysis on lysates of TAC-2 LacZ and TAC-2 DNTΔCT cells was repeated in panel C and demonstrates more clearly the presence of the DNTΔCT deletion protein.


A
aa
1
31
54
163
377
554
int-3
Met
TM
cdc10 repeat domain
HR
571
ΔNT
148
Met
424
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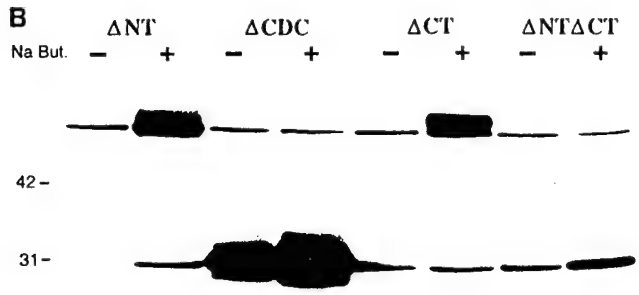
96
377
291
ΔCT
381
398
ΔNTΔCT
148
381
251
Number of amino acids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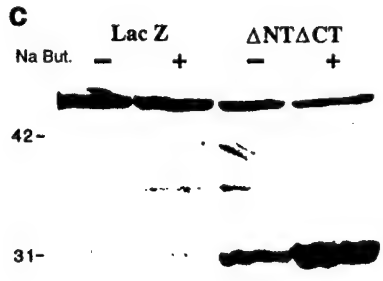
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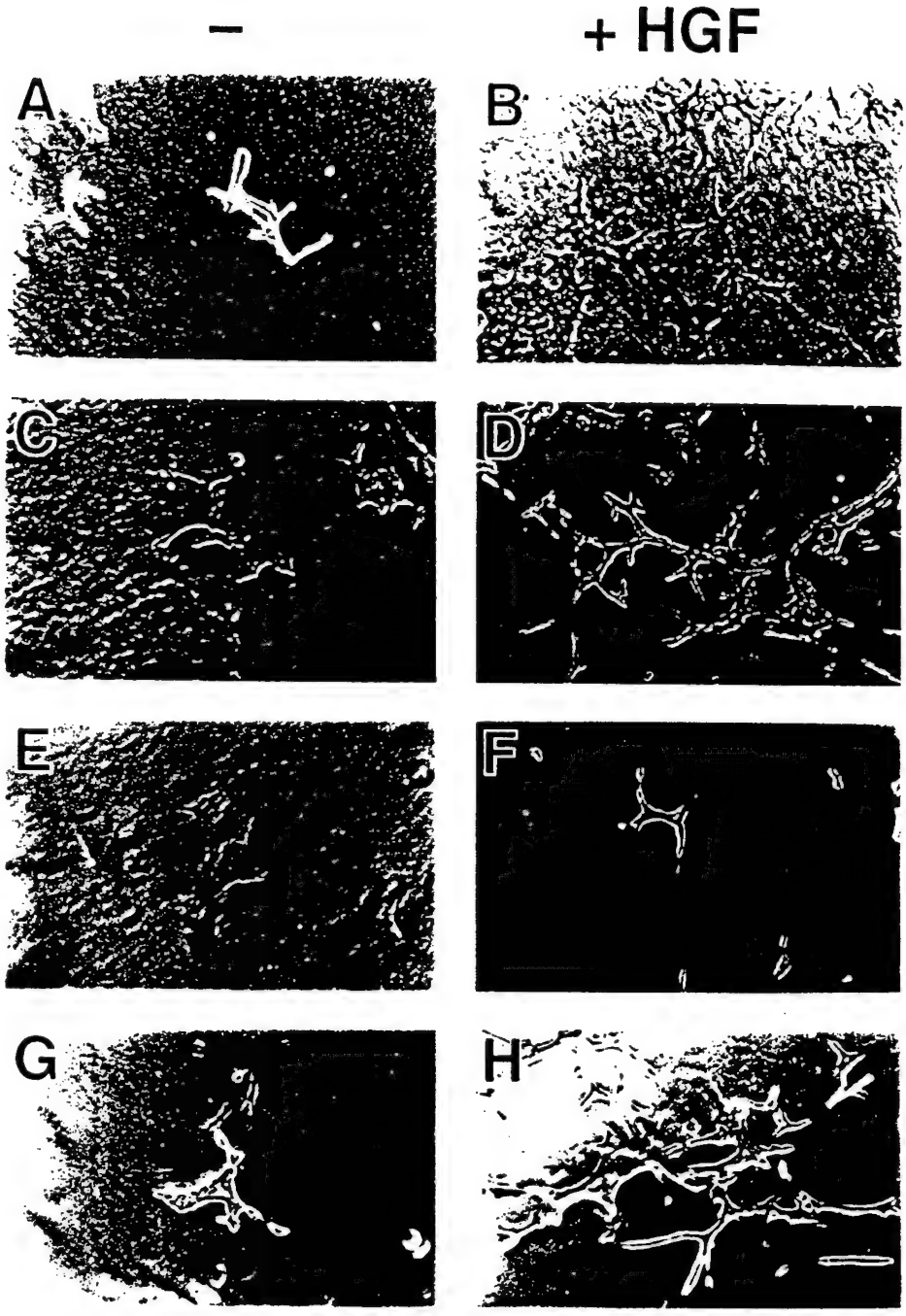
mutant int-3 cDNAs were HA-epitope tagged at their carboxy termini and TAC-2 cell lines programmed to express each deletion mutant were generated using the retroviral vector pLNCX. Immunoblot analysis using anti-HA monoclonal antibodies demonstrated expression of Notch4/int-3 deletion proteins of appropriate molecular weight in each respective cell line (Fig. 5B). The ΔNTΔCT Notch4/int-3 deletion protein with predicted molecular weight of 25 kD co-migrates with a non specific anti-HA antibody background band, however, can be detected upon longer exposure times or when these TAC-2 cells are treated with sodium butyrate (Fig. 5B). TAC-2 cell lines expressing the four different Notch4/int-3 deletion mutants were grown in collagen gels as described above, and the ability of each Notch4/int-3 deletion mutant to inhibit HGF induced branching morphogenesis of TAC-2 cells was analyzed. As shown in Fig. 6, TAC-2 cells expressing either ΔNT (Fig. 6A and 6B), ΔCDC (Fig. 6C and 6D) or ΔNTΔCT (Fig. 6G and 6H) are responsive to HGF induced branching morphogenesis. In contrast, when grown in the presence of HGF, ΔCT expressing TAC-2 cells (Fig. 6E and 6F) display an identical phenotype as the TAC-2 int-3 cells. Hence, the carboxy terminus of the Notch4/int-3 is not required for Notch-mediated inhibition of TAC-2 branching morphogenesis. Thus, in this assay the activity of the Notch4/int-3 oncoprotein can be conferred by the amino terminus and cdc10 repeats.

Branching morphogenesis in cells co-expressing Wnt-1 and Notch4/int-3 oncoproteins.

The activation of the Wnt-1 and Notch signaling pathways resulted in opposite effects on HGF- or TGF-β2-induced branching morphogenesis of TAC-2 cells. To explore the interactions between these two signaling pathways, we investigated the effect of simultaneous expression of both Wnt-1 and Notch4/int-3 proteins on TAC-2 branching morphogenesis. The above described TAC-2 LacZ and TAC-2 int-3 cell lines, which were generated with the pLNCX expression vector, were now also programmed to

Fig. 6 TAC-2 cell ductal morphogenesis assay with int-3 mutants. TAC-2 cells programmed to express ΔNT (A,B), ΔCDC (C,D), ΔCT (E,F), and ΔNTΔCT (G,H) were grown in collagen gels either in the absence of exogenous growth factor (A,C,E,G), or in the presence of HGF (B,D,F,H). HGF induces branching morphogenesis of TAC-2 ΔNT cells (B), TAC-2 ΔCDC cells (D) and TAC-2 ΔNTΔCT cells (H) TAC-2 ΔCT cells fail to undergo branching morphogenesis when grown in the presence of either HGF (F).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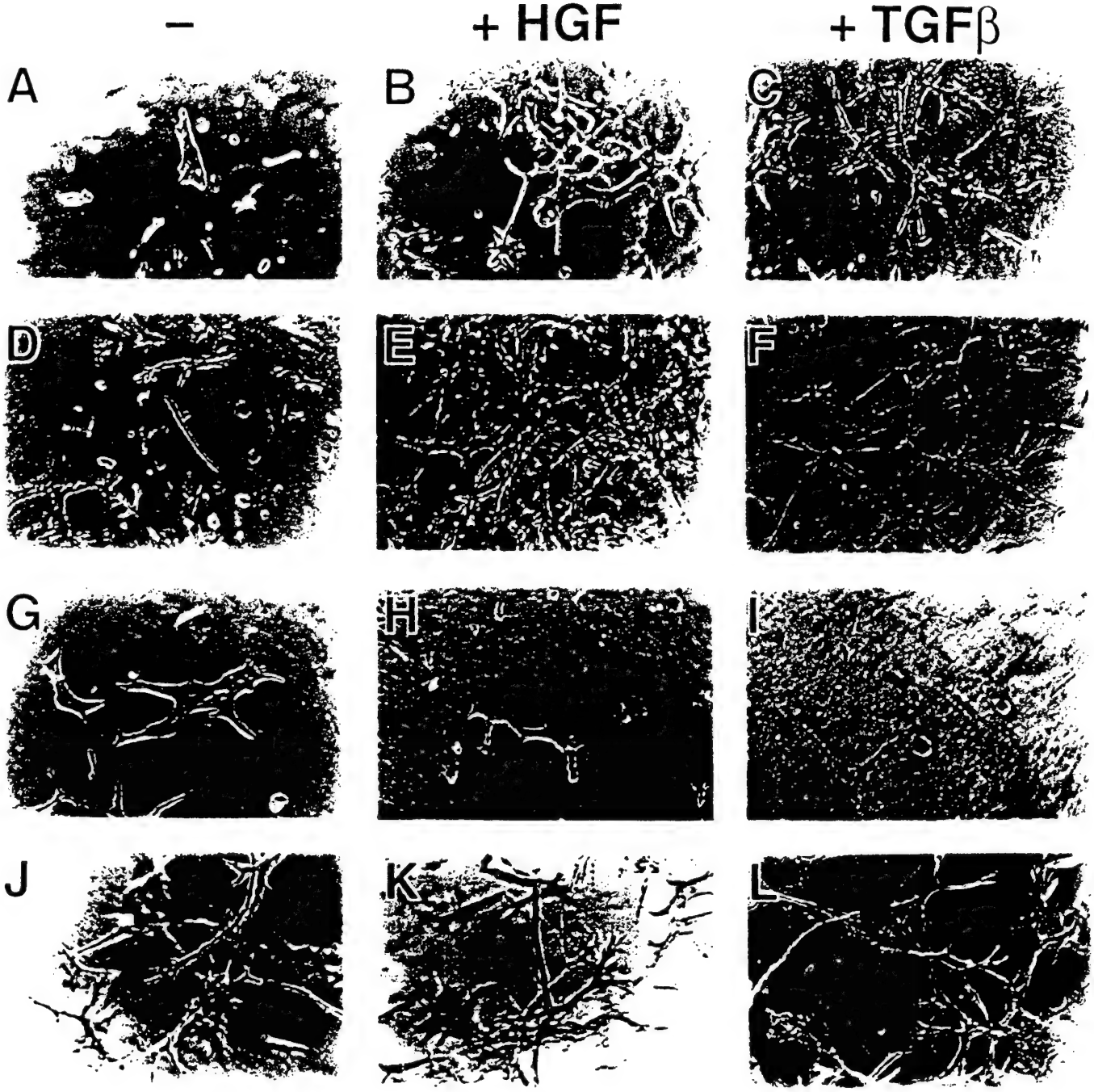
express Wnt-1 using the retroviral vector pLHTCX. This vector drives gene expression from the CMV promoter and contains the hygromycin resistance gene. In this fashion, four additional TAC-2 cell lines were generated that were named TAC-2 LacZ/ctr, TAC-2 LacZ/Wnt-1, TAC-2 int-3/ctr and TAC-2 int-3/Wnt-1 (where ctr denotes control empty pLHTCX vector). To determine appropriate protein expression in each of these four cell lines, immunoblot analysis showed Notch4/int-3 and Wnt-1 proteins were expressed as expected and at levels similar to those previously found to confer activity (data not shown). Each of the four cell lines were grown in collagen gels to determine their ability to undergo HGF- or TGF-β2-induced branching morphogenesis (Fig. 7). This assay was repeated three times with similar results. Doubly infected control cells TAC-2 LacZ/ctr (Fig. 7A, B, C) remained responsive to both HGF and TGF-β demonstrating that two rounds of drug selection did not affect the phenotype of the TAC-2 cell lines. As observed previously for TAC-2 Wnt-1 cells, TAC-2 LacZ cells that are now programmed to express Wnt-1 (Fig. 7D, E, F) form small colonies that undergo modest branching even in the absence of HGF or TGF-β2; these cells form extensive elongated branches when grown in the presence of HGF or TGF-β2. The activity found for Notch4/int-3, that is the inhibition of HGF- and TGF-β-induced branching morphogenesis, was also found in the TAC-2 int-3/ctr cell line (Fig. 7G, H, I). Wnt-1 and Notch4/int-3 co-expressing cells, TAC-2 int-3/Wnt-1, are able to form colonies displaying branching and elongation and have an appearance similar to that of TAC-2/LacZ/Wnt-1 cells (Fig. 7J). An examination of several fields reveal that TAC-2 int-3/Wnt-1 cells displayed increased responses when treated with either HGF or TGF-β, thus these cells now regain responsiveness to these factors (Fig. 7K, L). Our results indicate that Notch activation attenuates responsiveness of TAC-2 cells to both HGF and TGF-β and that Wnt-1 can override the Notch activity in TAC-2 cells.

Fig. 7 TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ/ctr (A,B,C), LacZ/Wnt-1 (D,E,F), int-3/ctr (G,H,I), or int-3/Wnt-1 (J,K,L) were grown in collagen gels either in the absence of exogenous growth factor (A,D,G,J), in the presence of HGF (20 ng/ml)(B,E,H,K), or in the presence of TGF-β2 (50 pg/ml)(C,F,I,L). HGF and TGF-β2 induce branching morphogenesis of TAC-2 LacZ/ctr cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 LacZ/Wnt-1 cells in the absence of either HGF or TGF-β2 (D), and robust branching is observed when TAC-2 LacZ/Wnt-1 cells are grown in the presence of either HGF (E) or TGF-β2 (F). TAC-2 cells programmed to express int-3/ctr fail to undergo branching morphogenesis when grown in the presence of either HGF (H) or TGF-β2 (I). TAC-2 cells programmed to express both int-3 and Wnt-1 undergo branching morphogenesis in the absence of exogenous growth factor (J), and form a robust branching network when grown in the presence of HGF (K) or TGF-β2 (L) in a similar manner when compared to TAC-2 cells programmed to express Wnt-1 solely (D,E,F).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Discussion

In this study, we have detailed a regulatory hierarchy involved in the branching morphogenesis of TAC-2 mammary epithelial cells. This regulation includes four distinct signaling pathways; the Wnt, Notch, HGF, and TGF-β signaling cascades. Using an *in vitro* model that reflects the branching morphogenesis exhibited during mammary gland development we have assessed the potential interactions between several different signaling pathways. This approach has allowed us to establish the relationships between these pathways. One remarkable feature of the regulation of branching morphogenesis we describe is its similarity to the regulatory pathways leading to morphogenetic events during *Drosophila* development.

Wnt proteins as branching morphogens in the mammary gland.

Formation of branching cords is induced in collagen gel cultures of TAC-2 cells by the addition of either HGF or low concentrations of TGF-β. Wnt-1 proteins induce moderate branching and elongation of TAC-2 cell tubules in the absence of added HGF or TGF-β2. The extent of Wnt-1 induced branching morphogenesis of TAC-2 cells is comparable to the induction by either HGF or TGF-β2. Our evidence suggests that Wnt-1 acts as a morphogen in this capacity. First, Wnt-1 induces a change in a morphogenetic event, the formation of branched epithelial tubules or cords. Second, Wnt-1 does not appear on its own to alter the growth properties of the TAC-2 cells. Finally, Wnt-1 can induce branching in an environment where cysts typically form; that is, in hydrocortisone and cholera toxin treated cultures. Such cultures form spheroidal cysts enclosing a widely patent lumen. In this environment, peptide growth factors that display mitogenic and not morphogenetic properties would increase the size of the cyst but the spheroidal structure would be maintained. In contrast, Wnt-1 alters the morphogenetic behavior in such a way that new branch points are formed and the structures take on a tubular morphology.

Several Wnt proteins are expressed in the mammary gland during periods of morphological changes of the ductal epithelium (Gavin and McMahon, 1992; Weber-Hall et al., 1994). Ectopic expression of Wnt-1 *in vivo* suggests a role for Wnt proteins in cell proliferation during mammary gland development; however, morphogenetic changes also occur in response to Wnt-1. It is has been proposed that the Wnt-1 expression mimics the activity of an endogenous mammary gland Wnt proteins. A transgenic line driving expression of *Wnt*-1 to the mammary gland displays a hyperplastic phenotype, indicative of increased proliferation (Tsukamoto et al., 1988). In addition, both virgin females and males display a marked increase in the number of terminal branches, and in fact resemble the hormonally stimulated glands normally observed in pregnant animals. Tissue reconstitution experiments in which *Wnt*-1 is ectopically expressed in mammary epithelium also result in a hyperplastic gland where duct epithelium show abundant fine side-branches, suggesting that Wnt-1 may instruct the epithelium to form branches (Edwards et al., 1992). This phenotype is most likely not simply a consequence of proliferation as it is not seen with a variety of other oncogenic proteins which when ectopically expressed in the mammary gland induce hyperplasia without increasing branching (Edwards, 1993).

The Wnt signal transduction pathway is mediated in part through β-catenin, a protein associated with cadherins, and which is necessary for the adhesive functions of adherens junctions (Miller and Moon, 1996). Wnt-1 signaling results in stabilization of the cytoplasmic pool of β-catenin (Lin et al., 1997; Shimizu et al., 1997), which then can associate with downstream targets in the cytoplasm to transduce signals that lead to regulation of target gene expression (Molenaar et al., 1996; Morin et al., 1997). TAC-2 cells programmed to express Wnt-1 in fact display increased levels of cytosolic β-catenin, when compared to TAC-2 LacZ cells (our unpublished data). This stabilization may be

regulated by the phosphorylation of β-catenin on serine/threonine residues, possibly by glycogen synthase kinase 3 (GSK-3) (Yost et al., 1996). Recent evidence has demonstrated the importance of β-catenin/cadherin interactions in regulating cell adhesion, cell migration and epithelial phenotype in embryonic development (Gumbiner, 1996). The activation of β-catenin by Wnt-1 induced signaling may result in changes of the adhesive and migratory characteristics of mammary epithelial cells and consequently affect ductal morphogenesis of TAC-2 cells.

Cooperative interactions between Wnt-1 and HGF or TGF-β.

In response to the combined effects of Wnt-1 and HGF, TAC-2 cells form a network of elongated and branching tubules that is far more extensive than the branching cords observed when TAC-2 cells are grown in the presence of Wnt-1 or HGF singularly. We propose that the combined effect of HGF addition and Wnt-1 expression is not the result of the sum of their independent activities on branching morphogenesis, but that Wnt signaling synergizes with the HGF/c-met tyrosine kinase pathway. The possibility that Wnt proteins cooperate *in vivo* with the HGF/c-met pathway in the regulation of mammary morphogenesis is supported by the overlapping temporal patterns of *Wnt* genes and HGF/c-met expression (Gavin and McMahon, 1992; Pepper et al., 1995; Weber-Hall et al., 1994).

One potential area where these two signaling pathways could converge might be through their effects on the catenin and cadherin proteins. The cooperation between Wnt-1 and HGF may be explained by their combined activation of β-catenin. β-catenin has been detected in a complex with the EGF receptor and can be phosphorylated in response to EGF and HGF (Hoschuetzky et al., 1994; Shibamoto et al., 1994). In addition, the Ras pathway is essential for the biological activity induced by HGF/c-met (Hartmann et al., 1994) and β-catenin has been demonstrated to be a substrate for tyrosine kinases and to

become tyrosine phosphorylated in cells expressing activated Src and Ras (Behrens et al., 1993). Another catenin-like protein, p120, which was identified as a substrate of Src and several receptor tyrosine kinases, interacts with the cadherin-β-catenin complex and may participate in regulating the adhesive function of cadherins (Daniel and Reynolds, 1995). EGF is also able to stimulate branching morphogenesis of TAC-2 cells, although not to the same extent as HGF, whereas NGF, bFGF, IGF-II and KGF can not (Soriano et al., 1995). These activities correlate with the reported phosphorylation of β-catenin by the EGF and HGF signal transduction pathways (Shibamoto et al., 1994). It is yet unclear how tyrosine phosphorylation of β-catenin might regulate its activity. Tyrosine phosphorylated β-catenin is found in a detergent soluble pool (Hoschuetzky et al., 1994; Kinch et al., 1995), which may reflect specific phosphorylation of a free pool of β-catenin. Since both Wnt-1 and HGF signaling can converge on β-catenin, it is therefore possible that the observed cooperation between HGF and Wnt-1 is due to their combined action on β-catenin activity.

Wnt-1, HGF, and TGF-β could induce branching morphogenesis by regulating the adhesive and migratory properties of TAC-2 cells through modulation of extracellular matrix components and their interaction with their receptors. Since HGF has been demonstrated to decrease adhesion of TAC-2 cells to collagen and to enhance the deposition of type IV collagen it is also possible that the observed cooperation between HGF and Wnt-1 is due to their combined effect on cell-substrate adhesion. TGF-β signaling involves receptors with serine/threonine kinase activity which are known to regulate the synthesis and degradation of extracellular matrix molecules and to induce matrix organization. Induction of branching morphogenesis by TGF-β could be mediated by a remodeling of extracellular matrix components and cell-substrate interactions. The Wnt signal transduction pathway may also regulate cell-substrate interactions, and the

combined activity of both Wnt-1 and TGF-β may explain their cooperative activities on the branching morphogenesis of TAC-2 cells.

In *Drosophila*, wingless (*wg*) and the TGF-β homologue *Decapentaplegic* (*Dpp*) have been shown in some cases to act in combination to regulate gene transcription during inductive events. In particular, Wg and Dpp have been shown to act in combination during limb development (Campbell et al., 1993; Diaz-Benjumea et al., 1994) and to induce *Ultrabithorax* expression during endoderm induction (Riese et al., 1997). Wnt-1 and TGF-β signaling may similarly converge to affect gene transcription during branching morphogenesis in the mouse mammary gland.

Notch inhibits branching morphogenesis of mammary epithelial cells.

We demonstrate that Notch activation inhibits both the HGF and TGF-β induced branching morphogenesis of TAC-2 mammary epithelial cells. The precise mechanism of this inhibition is unclear. Activation of Notch signaling has been demonstrated to inhibit or alter the cell fate commitment or differentiation of a variety of different cell types (Artavanis-Tsakonas et al., 1995; Greenwald and Rubin, 1992). For instance, *C. elegans* Lin-12 controls cell fate decisions during gonadogenesis, *Drosophila Notch* acts to control cell fate during neuroblast and photoreceptor cell differentiation, an activated *Xenopus Notch* can affect epidermal and neural crest cell development, and an activated mouse *Notch*1 can control cell fate during myogenesis and neurogenesis of cultured mouse cells (Artavanis-Tsakonas et al., 1995; Greenwald and Rubin, 1992). Transgenic mice that use the MMTV viral promoter to express the Notch4/int-3 oncoprotein, the activated form of Notch4, display severely impaired mammary ductal growth (Jhappan et al., 1992). When *Notch4/int-3* is expressed from the whey acidic protein promoter, whose expression is restricted to the secretory mammary epithelial cells, the differentiation of the secretory lobules of the transgenic animals is profoundly inhibited

(Gallahan et al., 1996). These experiments demonstrate that *Notch4/int-3*, like many other activated *Notch* genes, can act as a regulator of cell fate decisions in the mammary gland of mice. Little is known about the spatial and temporal pattern of *Notch* gene expression in the mammary gland, however, *Notch4* is expressed *in vivo* in the murine mammary gland (Sarkar et al., 1994; Smith et al., 1995). *Notch* genes may thus regulate the cell fate decisions occurring during mammary gland development that lead to the branched epithelial strutcture of the gland.

We have found that Notch activation can affect the response of TAC-2 mammary epithelial cells to either HGF or TGF-β. Since HGF acts through a tyrosine kinase receptor and TGF-β acts through serine/threonine kinase receptors, the affects of Notch activation may involve more than specific inhibition of a particular signaling cascade. Notch may regulate the competency of TAC-2 cells to respond to several different factors, possibly by shifting TAC-2 cells to a fate that is not predisposed to undergoe branching morphogenesis. This model would be consistent with the proposed activites of Notch proteins in several different organisms. Alternately, these signaling pathways may be controlled by Notch at a point at which they may converge to induce expression of genes important for branching morphogensis. Recently, the intracellular domain of LIN-12, a *C. elegans* Notch, has been demonstrated to associate with EMB-5, which encodes for a cytoplasmic protein containing a SH2 domain (Hubbard et al., 1996). This finding raises the possibility that the Notch signaling proteins may interact directly with those elicited by tyrosine kinase receptors, such as the HGF receptor (c-met).

We have demonstrated that the domain, carboxy terminal to the cdc10 repeats, of the Notch4/int-3 oncoprotein is not required for biological activity. However, the amino terminal domain and the cdc10 repeats are required for Notch4/int-3 activity. These findings are consistent with previous observed data for other *Notch* genes. The RAM23

domain which is localized between the transmembrane and cdc10 repeats has been demonstrated to be the binding site of CBF-1, a downstream and essential element in Notch signaling (Hsieh et al., 1996). Deletion of the amino terminal domain of Notch4/int-3, which contains the RAM23 domain, may eliminate binding to CBF-1, and hence destroy Notch4/int-3 activity. The region of the LIN-12 protein that includes the RAM-23 domain and cdc10 repeats appears to interact with another downstream and positive regulator, EMB5 (Hubbard et al., 1996). Point mutations and deletions within the cdc10 repeats result in loss of function of Notch proteins (Greenwald, 1994). Our data thus indicates that Notch4 may interact and be regulated through similar mechanisms.

Competing influences of Wnt and Notch signaling in branching morphogenesis.

When TAC-2 cells are programmed to express both Wnt-1 and Notch4/int-3, the cells are able to undergo branching morphogenesis. In Wnt-1 and Notch4/int-3 coexpressing TAC-2 cells, branching morphogenesis can be increased by either HGF or TGF-β; that is, the cells regain responsiveness to these factors. The phenotype observed in Wnt-1 and Notch4/int-3 coexpressing cells was similar to that of TAC-2 cells expressing only Wnt-1. The opposite biological activities of Wnt-1 and Notch4/int-3 observed in the TAC-2 cell assay correlate well with the mammary gland phenotype observed in Wnt-1 and Notch4/int-3 transgenic mice that ectopically express these proteins in the mammary gland (Jhappan et al., 1992). **Although both oncogenes increase mammary tumor development, Wnt-1 stimulates a hyperplastic phenotype with increased ductal development whereas Notch4/int-3 inhibits ductal development.**

Wnt-1 can override the Notch4/int-3-mediated inhibition of branching morphogenesis providing the first evidence of interaction between these two signaling pathways in vertebrates. The dominance of Wnt-1 over activated Notch we have observed in murine

cells parallels the functional relationship proposed for *Drosophila Wnt* (*wingless*) and *Notch* during *Drosophila* development (Axelrod et al., 1996). In this study, genetic analysis suggests a pathway convergence between wingless and Notch signaling resulting in opposing effects during patterning of the developing *Drosophila* wing. Activation of the wingless signal leads to regulation of Notch activity, possibly by *Drosophila* dishevelled, a cytoplasmic protein that is also a positive mediator in the Wnt-1 signal transduction pathway. Analysis using a yeast interaction trap system demonstrated that Dishevelled physically associates with the intracellular domain of Notch. The antagonism between Wnt-1 and Notch4/int-3 seen in branching morphogenesis may also be mediated by common regulators of the two signaling pathways such as Dishevelled.

During mammary gland development, the growth and differentiation of the gland is regulated by mesenchymal-epithelial and epithelial-epithelial interactions. Cells often receive different signals simultaneously and must integrate them in order to take on the correct proliferative, morphogenetic or differentiative response. Notch inhibition of ductal morphogenesis may be an early event in ductal morphogenesis. An attractive mechanism for overcoming Notch and allowing ductal morphogenesis to initiate or progress would be to activate the expression of a *Wnt* gene(s). Wnt could then serve the dual function of suppressing Notch activity and initiating branching morphogenesis. Wnt signaling may then cooperate with other signaling pathways, such as those mediated by HGF and TGF-β, in order to complete branching morphogeneis. Our study thus has revealed complex interactions between the signal transduction pathways of Wnt, Notch, HGF and TGF-β, in regulating the branching morphogenesis of mammary epithelial cells.

Chapter 5

Notch4 and Jagged-1 induce microvessel differentiation of rat brain endothelial cells

Introduction

Notch4 is a member of the Notch/lin-12 family of transmembrane receptors that are involved in cell fate determination (Artavanis-Tsakonas et al., 1995; Greenwald, 1994). Like other Notch proteins, Notch4 extracellular domain is characterized by both Epidermal Growth Factor (EGF)-like repeats and lin-12/Notch repeats (LNR), and the intracellular domain contains ankyrin/cdc10 repeats (Uyttendaele et al., 1996). Analysis of invertebrate Notch/lin-12 mutants support a function for Notch/lin-12 receptors in intercellular signaling events that control cell fate. Mutants that delete the extracellular domain of *C. elegans lin-12* or *Drosophila Notch* result in dominant gain-of-function phenotypes. The int-3 form of Notch4, Notch4/int-3, was identified based on its oncogenic effects in the mouse mammary gland and encodes only the transmembrane and intracellular domain of Notch4 (Gallahan and Callahan, 1997; Gallahan et al., 1987; Uyttendaele et al., 1996). Notch4/int-3 behaves as a constitutively activated receptor (Gallahan et al., 1996; Jhappan et al., 1992; Uyttendaele et al., 1998). Similar activating mutations in the *Notch1* gene have been identified in T lymphoblastic leukemia and can lead to neoplastic transformation in vitro (Capobianco et al., 1997; Ellisen et al., 1991).

Drosophila Delta (Vassin et al., 1987) and *Serrate* (Fleming et al., 1990) and *C. elegans Lag-2* (Tax et al., 1994) and *Apx-1* (Mello et al., 1994) encode a family Notch/lin-12 ligands that are transmembrane proteins whose extracellular domains contain EGF-like repeats and a DSL domain (Delta-Serrate-Lag-2). Based on homology to the *Drosophila* ligands, Notch ligands have also been identified in vertebrates. *Jagged*-1, a rat homologue of *Drosophila Serrate*, contains the hallmarks of a Notch ligand (Lindsell et al., 1995). Other putative mouse Notch ligands Delta-like 1 (Bettenhausen et al., 1995) and Delta-like 3 (Dunwoodie et al., 1997) have been identified and are closely related to *Drosophila* Delta. More recently, a human *Jagged-2* gene has been identified (Luo et al.,

1997). Although several Notch ligands and Notch receptors have been identified in mammals, it is not clear if ligands display distinct specificity's towards different receptors.

Several genes encoding Notch ligands or receptors are expressed in the endothelium. *Notch4* is expressed primarily in endothelial cells of the vasculature of mouse embryos and adult tissues (Shirayoshi et al., 1997; Uyttendaele et al., 1996). Notch1 and Jagged-1 are also expressed in the endothelium, as well as a variety of other tissues (Franco Del Amo et al., 1992; Lindsell et al., 1996; Myat et al., 1996; Reaume et al., 1992). Recently, *Jagged-1* was identified as gene induced during angiogenesis *in vitro* (Zimrin et al., 1996). A role of Notch in vascular development is suggested by analysis of mice with targeted disruption of genes encoding Notch ligands or Notch regulatory components. Mice deficient in either Delta like-1 (Dll1) (Hrabe de Angelis et al., 1997; Wong et al., 1997) or Jagged-1 develop severe hemorrhages (T. Gridley and G. Weinmaster, unpublished data). Mice with a targeted disruption of the *Presenilin1* gene(Shen et al., 1997; Wong et al., 1997), which facilitates Notch activity, display a loss of expression of *Notch1*, *Dll1* and exhibit hemorrhages in the brain and spinal cord. Evidence for involvement of Notch genes in vascular disorders has come from an analysis of a human hereditary adult onset condition causing stroke and dementia. CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephelopathy) is a human disorder manifested by stroke and dementia and characterized by non-amyloid and non-artherogenic angiopathy of cerebral arterioles (Baudrimont et al., 1993). The CADASIL locus maps to the human *Notch3* gene and mutations in *Notch3* have been defined in CADASIL patients (Joutel et al., 1996). Thus, alterations in Notch signaling may disrupt development and integrity of the vasculature.

As Notch functions in cell fate regulation in *Drosophila* and *C. elegans*, Notch receptors and their ligands expressed in endothelium may similarly control cell fate decisions during vascular development. We used the Rat brain endothelial cell line, RBE4, to study the role of Notch in endothelial cell differentiation. Ectopic expression of an activated Notch4/int-3 or of Jagged-1 in RBE4 cells induced cellular structures with morphological and biochemical properties of endothelial microvessels. Thus, activation of the Notch signaling pathway can promote endothelial differentiation.

Results

Notch4 expression in kidney endothelial cells.

We previously described endothelial cell specific expression of Notch4 mRNA in mouse embryos and adult mouse lung, as analyzed by *in situ* hybridization (Uyttendaele et al., 1996). To define the pattern of Notch4 protein expression, we generated an anti-Notch4 rabbit antiserum directed against a GST fusion protein containing the carboxy-terminus of Notch4 (Hubbard et al., 1997). The presence of endogenous Notch4 protein was analyzed in mouse kidney sections, as the kidney contains characteristic glomeruli with endothelial cell clusters in the renal cortex. We first analyzed Platelet Endothelial Cell Adhesion Molecule-1 (PECAM), an extracellular protein specifically expressed in endothelial and platelet cells. Antibodies against PECAM were used as a positive control in these experiments as they stained glomeruli (Fig. 1B). As shown in Fig. 1D, anti-Notch4 antibody specifically detects glomeruli in the kidney cortex in a similar pattern as the anti-PECAM antibody (Fig. 1B). Thus, data presented here demonstrates endothelial expression of Notch4 protein in the kidney.

The anti-Notch4 antibody was used to detect ectopically expressed Notch4 and Notch4/int-3 proteins by immunoblot analysis (Fig. 2C). Transiently transfected 293 cells expressed either HA epitope-tagged or non-epitope-tagged versions of Notch4 and Notch4/int-3. Immunoblot analysis of extracts from these cells displayed the full length Notch4 and Notch4/int-3 proteins which migrate with an approximate molecular weight of 220 and 70 kD respectively (Fig. 2B and C).

RBE4 microvessel outgrowth is induced by activated Notch4 and Jagged-1.

Previous work has shown that the RBE4 cell line grown on collagen coated plates display a cobblestone morphology (Roux et al., 1994). When cultured in the presence of basic

Fig 1. Immunohistochemical analysis on adult mouse kidney sections. Endothelial cells within the cortical kidney glomeruli can be detected by using either an anti-PECAM antibody (B) or an antibody generated against the carboxy terminus of Notch4 (D). No anti- PECAM antibody was used in panel A, and both panel A and B were processed for horse radish peroxidase detection which results in a brown staining. Pre-immune serum at identical dilution was used in panel C, and both panel C and D were processed for alkaline phosphatase detection which results in a blue-purple staining. Panels C and D were counterstained with eosin.

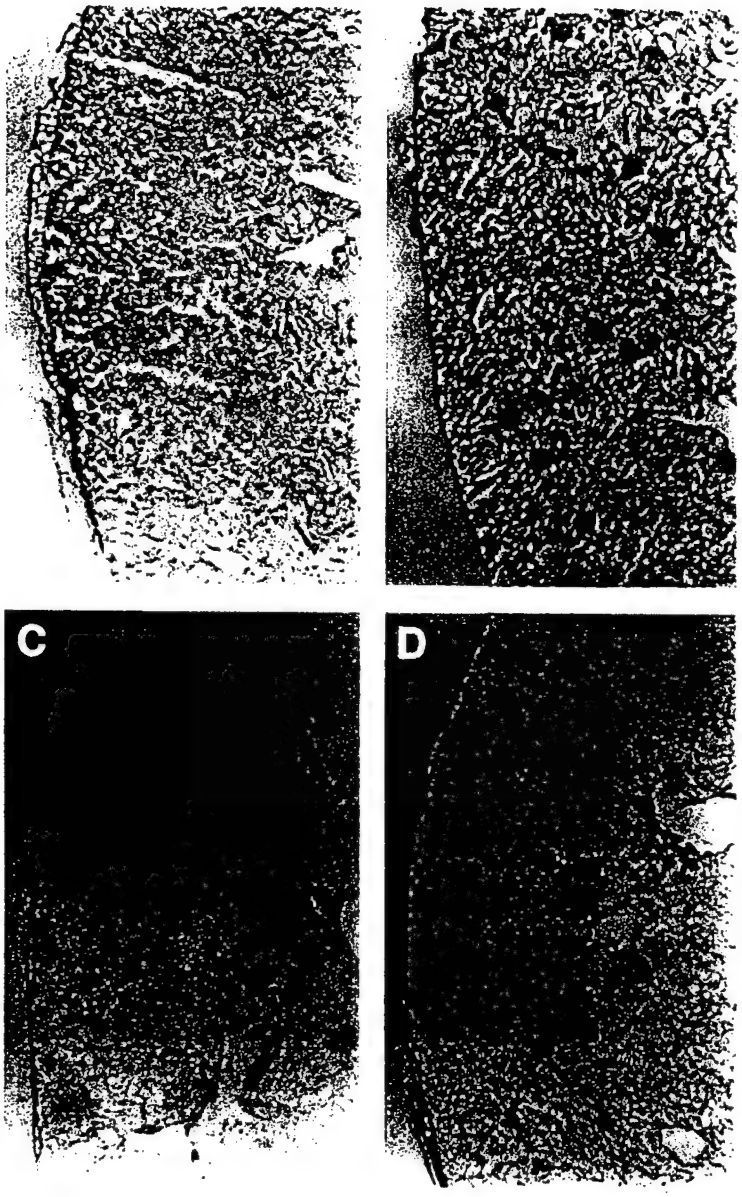
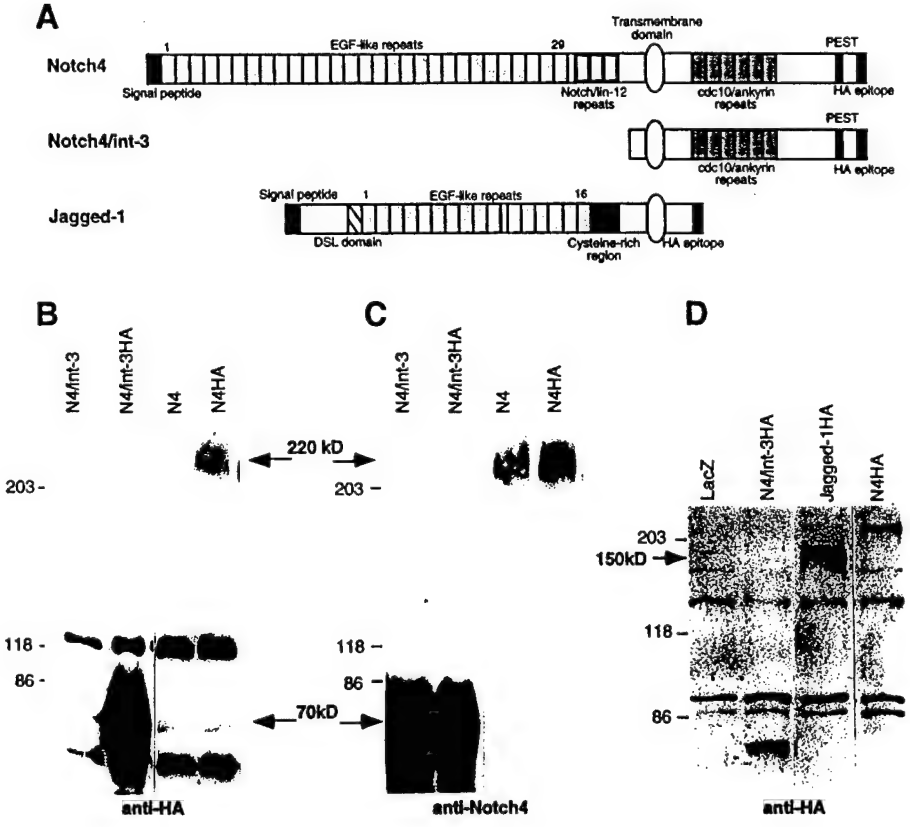

C
D

Fig 2. Schematic representation of the Notch4, Notch4/int-3 and Jagged-1 proteins (A). Conserved domains within each proteins are indicated. Imunnoblot analysis on lysates of 293 cells transiently transfected with either epitope tagged or non-epitope tagged cDNA's of Notch4/int-3 or Notch4 (B and C) using anti-HA antibody (B) or anti-Notch4 antibody (C). Immunoblot analysis on lysates of RBE4 cells programmed to express LacZ, Notch4/int-3, Jagged-1 or Notch4 using the anti-HA antibody, demonstrates expression of each respective protein (D).

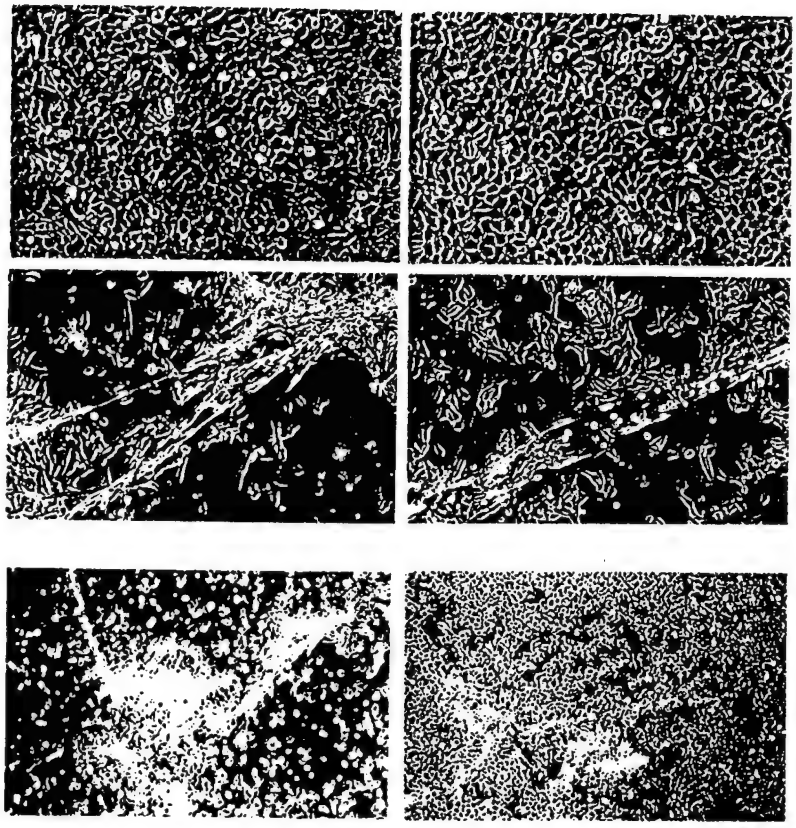

A
Notch4
1
EGF-like repeats
29
Transmembrane domain
PEST
Signal peptide
Notch/lin-12 repeats
cdc10/ankyrin repeats
HA epitope
Notch4/int-3
PEST
cdc10/ankyrin repeats
HA epitope
Jagged-1
Signal peptide
1
EGF-like repeats
16
DSL domain
Cysteine-rich region
HA epitope
B
N4/int-3
N4/int-3HA
N4
N4HA
220 kD
203 -
118 -
86 -
70kD
anti-HA
C
N4/int-3
N4/int-3HA
N4
N4HA
203 –
118 –
86 –
anti-Notch4
D
LacZ
N4/int-3HA
Jagged-1HA
N4HA
203 –
150kD
118 –
86 –
anti-HA

Fibroblast Growth Factor (bFGF), RBE4 cells change to a spindly morphology. When grown post-confluence, bFGF induces RBE4 cells to develop multi-cellular aggregates that arise from the monolayer. These three-dimensional aggregates consist of sprouts that extend above the monolayer and organize into curvilinear and bifurcating structures, resembling microvessels. bFGF-induced RBE4 microvessels contain high activity of alkaline phosphatase (ALP) and gamma glutamyl transpeptidase (γGTP), which are specific enzymatic markers for differentiated endothelium and brain capillaries respectively (Roux et al., 1994).

RBE4 cell lines were programmed to express the HA-tagged Notch4/int-3, Jagged-1 and LacZ proteins using the retroviral vector pLHTCX. RBE4 cell lines programmed to express Notch4 were generated by direct transfection of the HA-tagged *Notch4* cDNA. All three recombinant proteins were detected by immunoblot analysis of the respective RBE4 cell lines and migrated with their predicted molecular weight (220kD for Notch4, 70kD for Notch4/int-3, 150 kD for Jagged-1, see Fig. 2D).

The consequences of activating the Notch signaling pathway by ectopic expression of ligand (Jagged-1) or an activated receptor (Notch4/int-3) was analyzed in RBE4 cells. Control RBE4 cells expressing LacZ (RBE-LacZ) growth arrest at confluence and do not develop three dimensional structures (Fig. 3A). When confluent RBE-LacZ cells were grown with bFGF (5 ng/ml), they form modest sprouts and microvessels (data not shown). RBE-LacZ were indistinguishable from wild type RBE4 cells grown under identical conditions. RBE4 cells programmed to express either Jagged-1 (RBE-Jagged-1) or Notch4/int-3 (RBE-Notch4/int-3) develop sprouts and three dimensional capillary-like structures (Fig. 3C and D). These structures were not dependent on the presence of bFGF. These microvessel structures either float in the cell culture medium (although originating from a mount formed on the tissue culture plate) or are attached to the tissue

Fig 3. RBE4 cells that are programmed to express either LacZ (A) or Notch4 (B) display a cobble stone morphology when grown on collagen coated plates. RBE4 cells programmed to express Notch4/int-3 (C and E) or Jagged-1 (D and E) display a spindle shape morphology and spontaneously form microvessel structures. Structures were more prominent in RBE-Notch4/int-3 than in RBE-Jagged-1 cell cultures (compare E to F). Photographs A to D were taken at 100X magnification whereas photographs E and F were taken at 40X magnification.



culture plate. The extent of microvessel induction by Notch4/int-3 is far greater when compared to Jagged-1, as demonstrated in Fig. 3E and F. RBE4 cells programmed to express Notch4 (RBE-Notch4) do not form any microvessel structures (Fig. 3B). RBE-Notch4/int-3 or RBE-Jagged-1 cells displayed a more spindle shape morphology and do not form the cobblestone monolayer, as observed in RBE-LacZ or RBE-Notch4 (compare Fig. 3C and D with A or B). This morphological change is similar to when wild type RBE4 cells are treated with bFGF (Roux et al., 1994).

Microvessel structures induced by Jagged-1 and Notch4/int-3 in RBE4 cells were analyzed histochemically for the activities of two blood-brain barrier-associated enzymes, γGTP and ALP. These enzymes are specifically expressed in the brain vasculature, and their expression is induced by bFGF treatment of RBE4 cells (Roux et al., 1994). ALP activity was detected in microvessel structures induced by either Notch4/int-3 (Fig. 4B) or by Jagged-1 (data not shown). γGTP activity was also detected in both RBE-Jagged-1 (Fig. 4C) and RBE-Notch4/int-3 (data not shown). The detection of both ALP and γGTP activities was specific for microvessel structures and not found in surrounding monolayer cells. RBE4 cell lines that did not exhibit microvessels, RBE-LacZ and RBE-Notch4, did not display ALP or γGTP activities (data not shown).

Activation of the Notch signal transduction pathway in RBE4 cells.

Jagged-1 and Notch4/int-3 expression resulted in similar phenotypes in RBE4 cells, thus we hypothesized that RBE4 cells must express endogenous Notch receptors that can be activated by Jagged-1. Northern Blot analysis demonstrated endogenous expression of *Notch1*, *Notch3* and *Notch4* transcripts (Fig. 5A). We also found that RBE4 cells express endogenous *Jagged-1* transcripts (Fig. 5A). We could not make a statement about the relative levels of expression of *Notch* and *Jagged* transcripts as each lane in Fig. 5A utilized a different riboprobe.

Fig 4. Histochemical analysis of alkaline phosphatase (B) and gamma-glutamyl transpeptidase (C) activities in microvessel structures induced by RBE4 cells expressing Notch4/int-3 (B) or Jagged-1 (C). RBE4 cells surrounding the microvessel structures do not express either enzyme activity (B and C). Panel A is control.

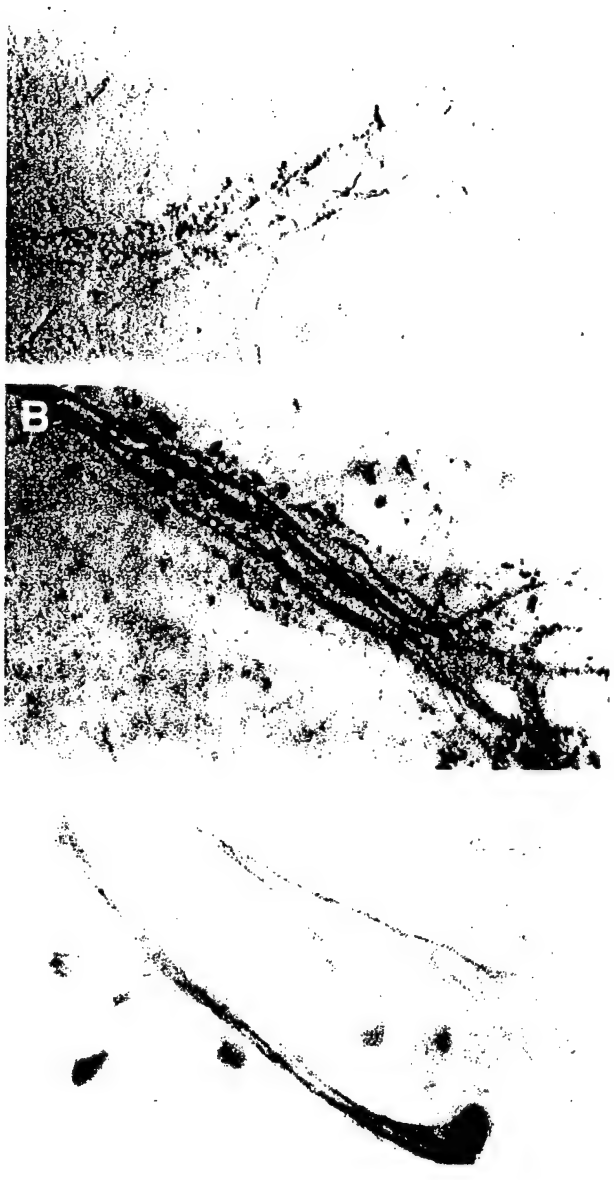
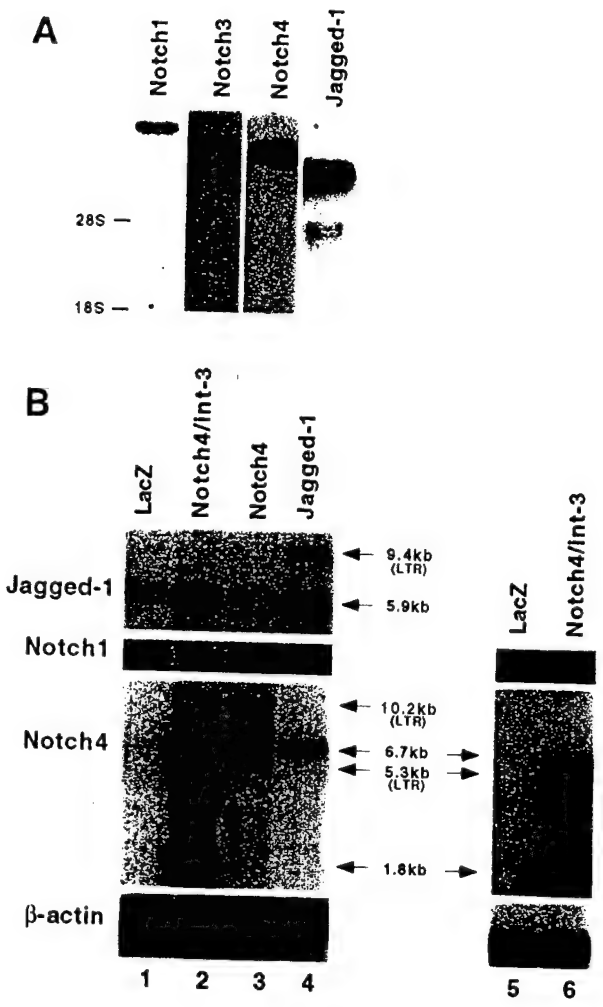

B

Fig 5. Northern blot analysis on RBE4 cells. Panel A, 40 mg total RNA from RBE4 cells was hybridized to riboprobes for either *Notch1*, *Notch3*, *Notch4* or *Jagged-1*. Panel B, 40 mg of total RNA (lane 1, 3, 5, 6) or 20 mg of total RNA (lane 2 and 4) from RBE4 cells programmed to express either LacZ, Notch4/int-3, Notch4 or Jagged-1, was hybridized to riboprobes for either *Jagged-1*, *Notch1*, *Notch4*, or *b-actin*. LTR-driven transcripts are denoted (LTR).


A
Notch1
Notch3
Notch4
Jagged-1
28S
18S
B
LacZ
Notch4/int-3
Notch4
Jagged-1
Jagged-1
Notch1
Notch4
β-actin
9.4kb (LTR)
5.9kb
10.2kb (LTR)
6.7kb
5.3kb (LTR)
1.8kb
LacZ
Notch4/int-3
1
2
3
4
5
6

Activation of Notch signalling has been reported to induce endogenous genes encoding Notch receptors and ligands (Luo et al., 1997). This induction can serve as a measure of signal activation and may lead to positive feedback control of *Notch* genes by their ligands (Wilkinson et al., 1994). To measure Notch signaling output in RBE4 cells, we assessed the level of endogenous *Notch* receptor and *Notch* ligand gene transcripts by northern blot analysis (Fig. 5B). RBE-Notch4/int-3 have increased levels of endogenous *Notch4* (6.7 kb) and *Jagged-1* transcripts (5.9 kb) (Fig. 5B, lane 2). The *Notch4*-specific riboprobe corresponds to the carboxy-terminal domain of *Notch4*, and can detect both ectopic *Notch4/int-3* transcripts (1.8 kb) and endogenous *Notch4* transcripts (6.7 kb). Notch4/int-3 expression in RBE cells specifically led to increased levels of endogenous *Notch4* transcripts (Fig. 5B, lane 2) and not endogenous *Notch1* (Fig. 5B, lane 2) or *Notch3* transcripts (data not shown). RBE-Jagged-1 cells also have increased levels of endogenous *Notch4* transcripts (Fig. 5B, lane 4). Again this activation is restricted to the endogenous *Notch4* gene since the levels of *Notch1* (Fig. 5B lane 4) and *Notch3* (data not shown) transcripts remain unchanged. We were not able to ascertain if Jagged-1 expressing RBE4 cells up-regulated endogenous *Jagged-1*, since both the ectopic and endogenous *Jagged-1* transcripts migrate with a similar molecular size (5.9 kb). RBE-Notch4 cells do not have increased levels of *Notch1* (Fig. 5B, lane 3), *Notch3* (data not shown) or *Jagged-1* transcripts (Fig. 5B, lane 3). The level of *Notch4* transcripts in these cells could not be evaluated since we can not distinguish by size between endogenous and ectopic *Notch4* transcripts.

RBE4 cell lines were generated with retroviral vectors designed to express genes via an internal CMV promoter; however, gene expression may also be driven by the retroviral LTR leading to expression of two different sized transcripts. CMV-driven transcripts correspond to 1.8 kb for *Notch4/int-3*, 6.7 kb for *Notch4* and 5.9 kb for *Jagged-1* (Fig. 5B). Larger transcripts encompassing the complete retroviral genome originate from the

LTR resulting in transcripts that are 3.5 kb larger than the CMV-driven transcripts. The LTR-driven transcripts are denoted in Fig. 5B (LTR) and correspond to 5.3 kb for *Notch4/int-3*, 10.2 kb for *Notch4* and 9.4 kb for *Jagged-1* (Fig. 5B). To clearly distinguish between the activated endogenous *Notch4* transcripts (6.7 kb) and the LTR-driven *Notch4/int-3* transcript (5.3 kb) a short exposure of a different northern blot from RBE-Notch4/int-3 cells is shown in Fig. 5B (lane 5 and 6). Fig. 5B lanes 2 and 6 both demonstrate that Notch4/int-3 activates expression of the endogenous *Notch4* gene (6.7 kb).

Discussion

To investigate the role of Notch4 and Jagged-1 in endothelial cell differentiation we used an angiogenic *in vitro* model system, the RBE4 cell line. Previous studies have demonstrated that bFGF, a well characterized angiogenic factor, induces microvessel formation and the expression of markers specific for either differentiated endothelium (ALP) or the blood-brain barrier (γGTP) (Roux et al., 1994). Ectopic expression of either Notch4/int-3 or Jagged-1 in RBE4 cells also results in the induction of microvessel structures that express both ALP and γGTP. Full length Notch4 expression did not elicit any observable phenotype. These studies suggest that Notch4/int-3 and Jagged-1 induce a biological activity in RBE4 cells that is similar to that induced by a known angiogenic agent bFGF. Thus, in rat brain endothelial cells, Notch activation results in an angiogenic response characterized by microvessel formation. Thus, Notch activation may regulate the angiogenic process in endothelium where Notch4 is found to be expressed, such as the vasculature of the adult kidney and lung.

Our data support the concept that Notch4/int-3 is a constitutive activated Notch allele as it induces a phenotype that is identical to when endogenous Notch receptors are activated by Jagged-1. We also note that ectopic expression of full length Notch4 in RBE4 cells does not result in Notch signal activation, thus receptor overexpression does not lead to constitutive receptor activation. It may also suggest that RBE4 cells do not express a Notch4 ligand. Alternately RBE4 cells may not express Notch4 ligands at levels sufficient to activate the ectopically expressed Notch4 receptor. The induction of microvessel structures by Notch4/int-3 appears to be more robust when compared to the induction by Jagged-1 suggesting that a constitutive activated form of the Notch4 receptor is more potent in its activity than a Notch ligand (Jagged-1).

Since Jagged-1 is able to induce a biological activity that is similar to Notch4/int-3, we hypothesize that RBE4 cells must express endogenous Notch receptors which can be activated by Jagged-1. Northern blot analysis demonstrated that the Notch1, Notch3 and Notch4 genes are expressed in RBE4 cells. Notch1 and Notch4 are known to be expressed in endothelium *in vivo* (Franco Del Amo et al., 1992; Lindsell et al., 1996; Myat et al., 1996; Reaume et al., 1992; Shirayoshi et al., 1997; Uyttendaele et al., 1996) and Notch3 may function in the vasculature (Joutel et al., 1996). RBE4 cells also express Jagged-1 when analyzed by Northern blot. Thus, although RBE4 cells express endogenous transcripts that encode for both Notch proteins and one of their ligands, they do not spontaneously form microvessel structures. This observation may suggest that the levels of Notch proteins or ligands in RBE4 cells are insufficient to activate the Notch pathway.

We demonstrated that the Notch signal transduction pathway is activated in RBE4 cells expressing either Notch4/int-3 or Jagged-1. Activation of Notch signaling results in up-regulation of the endogenous Notch4 and Jagged-1 transcripts. Thus, the observed microvessel induction of RBE4 cells correlated with Notch signal activation. Both Jagged-1 and Notch4/int-3 expression resulted in a similar up-regulation of the endogenous Notch4 gene but neither altered the levels of Notch1 and Notch3 transcripts. Activation of a particular Notch signaling pathway, in this case Notch4, led to transcriptional activation events that are specific for distinct endogenous *Notch* genes. The observation that Jagged-1 results in an identical transcriptional activation pattern as activated Notch4/int-3 may suggest that Jagged-1 is a Notch4 ligand.

Previous work has demonstrated that activation of the Notch signal transduction pathway in mammalian cells inhibits differentiative or morphogenetic events. For instance, activated Notch1 and Notch2 are able to inhibit myogenic differentiation(Hsieh et al.,

1997; Lindsell et al., 1995), and myeloid differentiation (Bigas et al., 1998), and activated Notch4 (Notch4/int-3) is able to inhibit branching morphogenesis of mammary epithelial cells (Uyttendaele et al., 1998). In contrast, we found that activated Notch4/int-3 promoted the differentiation of RBE4 cells. Cell fate determination may involve either inhibition or promotion of differentiative steps. Our data is thus a clear demonstration that Notch signal activation can regulate cell fate decisions that involve the promotion of differentiation in mammalian cells.

Cell fate and cell differentiation decisions likely play important roles during vascular development. Although some factors have been identified that regulate vascular growth, little is known about how these or other factors may regulate cell fate decisions (Hanahan, 1997). Our data suggest that Notch proteins and their ligands can promote differentiation and may be important in regulating cell fate decisions in the developing vasculature.

Chapter 6

Notch4 is required for endothelial cell development *in vivo*

Introduction

The development of the vertebrate vascular system proceeds through two mechanisms: vasculogenesis and angiogenesis. Vasculogenesis refers to the formation of blood vessels through the de novo differentiation of endothelial cells, while angiogenesis is the process by which blood vessels sprout from preexisting ones (Risau, 1997). During vasculogenesis, differentiating angioblasts associate into vessels in situ, or migrate to fuse with other angioblasts. The resulting vessels form a primary vascular plexus that then becomes remodeled when circulation begins, either by fusion to give rise to larger vessels or by regression and migration. The extraembryonic vessels in the yolk sac, the dorsal aorta, and the vascularization of organs that are of endodermal origin (lung, pancreas and spleen) develop by vasculogenesis (Pardanaud et al., 1989). The formation of new blood vessels by angiogenesis becomes more important later in embryogenesis, as smaller blood vessels sprout from larger ones, and in the adult during the processes of wound healing and tumor growth. The intersomitic arteries, for example, sprout from the dorsal aorta. In addition, organs that are of ectodermal origin, such as the brain, are vascularized by angiogenesis (Pardanaud et al., 1989).

Recently, a number of receptors and their cognate ligands have been implicated as important regulators of endothelial development. Vascular endothelial growth factor (VEGF) is a potent mitogen and chemoattractant for endothelial cells (Ferrara et al., 1992). The related receptor tyrosine kinases, Flk1 and Flt1, bind VEGF with high affinity (de Vries et al., 1992; Millauer et al., 1993; Quinn et al., 1993). Flk1 is expressed as early as 7.0 dpc in the presumptive mesodermal precursors of the blood islands, and then marks endothelial cells and their precursors during vasculogenesis and angiogenesis in the mouse (Shalaby et al., 1995; Yamaguchi et al., 1993). Flt1 expression can be detected approximately a half day later in gestation in the developing blood islands, and

goes on to mark angioblasts and endothelial cells (Fong et al., 1995). The role of VEGF and its receptors have been examined through the use of knockout mice. VEGF heterozygous and homozygous mutant embryos die by around 10.5 dpc, indicating that there might be a dose-dependent effect of VEGF during vascular development (Carmeliet et al., 1996; Ferrara et al., 1996). *VEGF+/-* embryos show poorly developed dorsal aortae at 8.5-9.5 dpc, with endothelial cells lining a much smaller lumen than normal. VEGF-/- embryos have a more severe phenotype, with only a few scattered *Flk1* and *Flt1* positive cells present at 8.5 dpc, and abnormally enlarged vascular structures instead of the normal dorsal aortae at 9.5 dpc. Embryos deficient for the VEGF receptor, Flk1, die between 8.5 and 9.5 dpc, lacking mature endothelial and hematopoietic cells (Shalaby et al., 1995). Examination of mutant embryos at 7.5 dpc reveals that they lack blood islands, suggesting that Flk1 is absolutely required for endothelial and hematopoietic differentiation (Shalaby et al., 1995). *Flt1* homozygous mutant embryos also die at the mid-somite stage, but these embryos do possess endothelial cells (Fong et al., 1995). The endothelial cells form abnormally enlarged vascular structures, with some endothelial cells present within the lumen of the larger vessels of the yolk sac and the embryo. The blood islands also appear disorganized at 7.5 dpc, with angioblasts intermixed with hematopoietic cells. Thus, Flt1 signaling may be important for endothelial cell-cell or cell-extracellular matrix interactions during development (Fong et al., 1995).

Two other receptor tyrosine kinases, Tie (also known as Tie1) and Tek (also known as Tie2), have similar extracellular domains and form a second subfamily of endothelial-specific receptor tyrosine kinases (Dumont et al., 1992; Partanen et al., 1992; Sato et al., 1993). These receptors are expressed later in endothelial development, at around 8.5 dpc in the embryo, and 7.5 dpc and 8.0 dpc in the yolk sac for the Tek and Tie receptors, respectively (Dumont et al., 1995). Embryos that are homozygous mutant for *Tek* die around 9.5 dpc with fewer endothelial cells and hemorrhaging throughout the embryo.

Two ligands for Tek have recently been identified, angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) (Davis et al., 1996; Maisonpierre et al., 1997). *Ang1-/-* embryos die around 12.5 dpc, with a phenotype that is strikingly similar to that of *Tek-/-* embryos (Suri et al., 1996). These embryos have underdeveloped, less complex vessels and more immature endocardium when compared to wildtype embryos. The *Tie* gene has also been knocked out by homologous recombination in ES cells (Puri et al., 1995; Sato et al., 1995). *Tie-/-* embryos die due to hemorrhaging and edema at either 14.5 dpc, or as neonates depending on the mouse strain backgrounds (Puri et al., 1995; Sato et al., 1995). The Tie receptor does not appear to be essential for hematopoiesis, vasculogenesis or early angiogenesis, but plays an important role in the integrity and survival of endothelial cells during later angiogenesis. Thus, in short, Flk1 signalling is required at the earliest stages of endothelial cell differentiation, and Flt1 is necessary for the cell-cell interactions essential for the formation of endothelial tubes. Tek and its ligands, Ang1 and Ang2, are involved in the regulation of vessel integrity during normal growth and development, angiogenesis and vessel regression. Finally, Tie1 appears to be required for late angiogenesis during embryogenesis.

The identification and characterization of endothelial specific RTKs has led to an emerging picture of how these molecules are involved in various aspects of vasculogenesis and angiogenesis. It would be of interest to identify other factors that play a role in these processes. One candidate is the mammalian Notch4 protein, which is specifically expressed in endothelial cells and may regulate cell fate decisions in endothelial cell development(Uyttendaele et al., 1996). *Notch4* expression has been observed as early as 7.5 dpc, by RT-PCR (Shirayoshi et al., 1997). Whole mount in situ analysis has detected *Notch4* by 8.5 dpc, and then onwards throughout embryogenesis in endothelial cells, in a pattern similar to that observed for *Flk1* (Shirayoshi et al., 1997). The mammalian Notches, *Notch1* and *Notch3*, are also expressed in endothelial cells, and

at least one vertebrate Notch ligand has been reported with an endothelial expression pattern, chick *Serrate-1* (Myat et al., 1996). Since the *Notch4* null mutant has no phenotype (Thomas Gridley, unpublished data), we have analysed the effect of expressing a gain of function mutation of the Notch4 protein (Notch4/int-3) in endothelial cells *in vivo*.

Results

Generation of Flk1/int-3 ES cells

A targetting vector was constructed that contains *int3HA* (the activated *Notch4* that was epitope tagged as previously described) flanked by *Flk1* homology, and the neomycin and thymidine kinase cassettes for positive and negative selection, respectively. This construct was electroporated into *Tie*+/- ES cells, and correctly targetted clones (Flk1/int-3) were positively selected for using G418 and negatively selected for using gancyclovir. *Tie*+/- ES cells were chosen for these experiments, which have one allele of the *Tie* gene replaced by the LacZ gene. Since *Tie* is an endothelial cell specific gene, β-galactosidase staining was used to evaluate the vasculature in embryos derived from the Flk1/int-3 ES cells. Replacement (with the β-galactosidase gene) of one allele of the *Flk1* and the *tie* gene simultaneously, did not result in any observable phenotype (data not shown). Thus, targetted ES cell clones will express Notch/int-3 under the transcriptional control of the *Flk1* promoter, and will express LacZ under the transcriptional control of the *Tie* promoter. The expression of β-galactosidase was used as a marker for endothelial cells. Southern blot hybridization was done to determine ES cell clones that had one *Flk1* allele replaced with *Notch/int-3* by homologous recombination (data not shown). Two clones were isolated and named 2G6 and 1A12. As control, one clone (2G5R) was used that incorporated Notch4/int-3 by non-homologous recombination, as determined by Southern blot analysis (data not shown).

Generation of Flk1/int-3 ES embryos

The *flk1/int3* ES cell lines were used in aggregations with tetraploid embryos. This technique is useful to analyze dominant phenotypes. Two-cell diploid embryos are fused to become a tetraploid one-cell embryo, and aggregated with ES cells. The tetraploid cells fail to contribute to embryonic tissues, and contribute to the visceral endoderm and

extraembryonic ectodermal tissues. Thus, aggregation of ES cells and a tetraploid embryo produces embryos that are wholly ES cell derived. Flk1/int-3 and control Tie+/- embryos were generated using the above described technique. Flk1/int-3 embryos die between days 9.5 and 10.5 dpc. The vasculature in Flk1/int-3 and control embryos was analyzed by β-galactosidase staining, first in whole mounts (Fig. 1) and then in histological sections of these embryos (Fig. 2). The extent of the developing vasculature in mutant embryos appears to be somewhat restricted, and fewer small vessels are observed in mutant embryos (Fig. 1). The major blood vessels such as the dorsal aorta and cardinal veins are present in mutant embryos and embryonic blood cells are present in blood vessel lumens (Fig. 2). It appears that many of the bloodvessels in mutant embryos are dilated and the integrity of the blood vessel wall is lost. Around these dilated vessels, large areas of necrosis are seen in mutant embryos (Fig. 2).

Analysis of Flk1/int-3 embryoid bodies

To determine whether the above described phenotype is the result of an inherent defect in endothelial cells or whether the vascular phenotype is secondary to an unknown but unrelated defect, embryoid bodies (EB) were generated from the ES cell lines to investigate the development of the endothelium. The Flk1/int-3 cell lines (2G6 and 1A12) were compared with the parental *Tie+/-* cell line (ID6) and to an ES cell line that received the *int-3* gene through non-homologous recombination (2G5R) in their ability to produce vascular structures. Analysis of the vascular networks in the EB was done by in situ β-galactosidase staining as described above. As shown in Figs. 3 and 4, vascular networks observed in EB that express Notch4/int-3 (Fig. 3C and D) are disorganized and do not form the fine and homogenous vascular networks which are observed in control embryoid bodies (Fig. 3A and B). These data thus suggest that the expression of Notch4/int-3 disrupt vascular development and this phenotype is the result of an inherent defect in endothelial cells expressing a constitutive activated Notch4 protein.

Fig. 1 Control Tie +/- (A and C) and Flk-1/int-3 (B and D) embryos at 8.5 (A and B) and 9.5 (C and D) dpc were processed for whole mount β-galactosidase staining to visualize the endothelial cells. No differences in the vasculature are observed between control and mutant embryos at 8.5 dpc (compare A and B). At 9.5 dpc, vasculature in mutant embryos appears to be more restricted and disorganized (compare C and D).

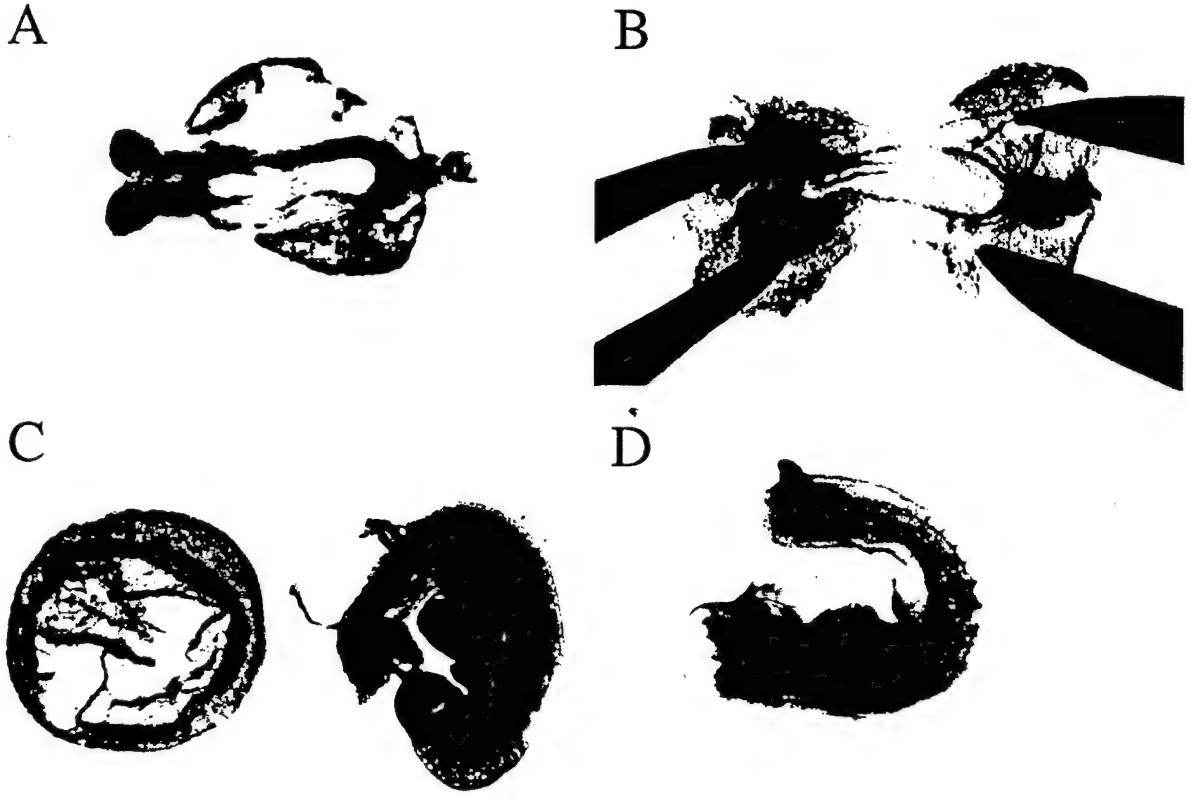

A
B
C
D

Fig. 2 Histological sections of Flk-1/int-3 embryos at 9 dpc. Panel A, shows a cross-section through the spinal cord, cardinal vessels and heart. Panel B, shows a cross-section through the brain. Enlarged and collapsed vessels are observed and large areas of necrosis are seen.

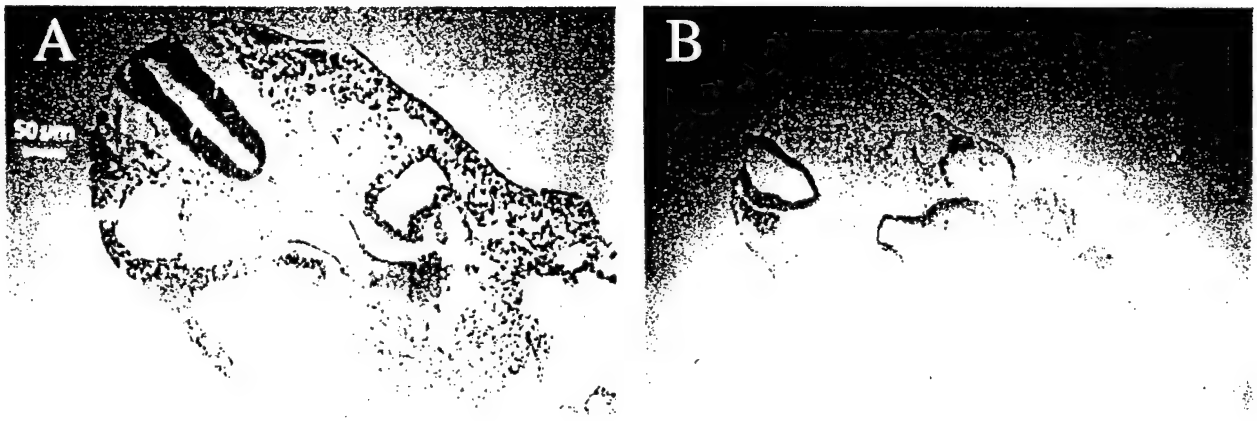

A
50 μm
B

Fig. 3 Embryoid bodies (40X magnification) were generated from either Tie+/- (A), 2G5R (B), 2G6 (C) or 1A12 (D) ES cells. The endothelial cells in embryoid bodies were visualized by β-galactosidase staining.

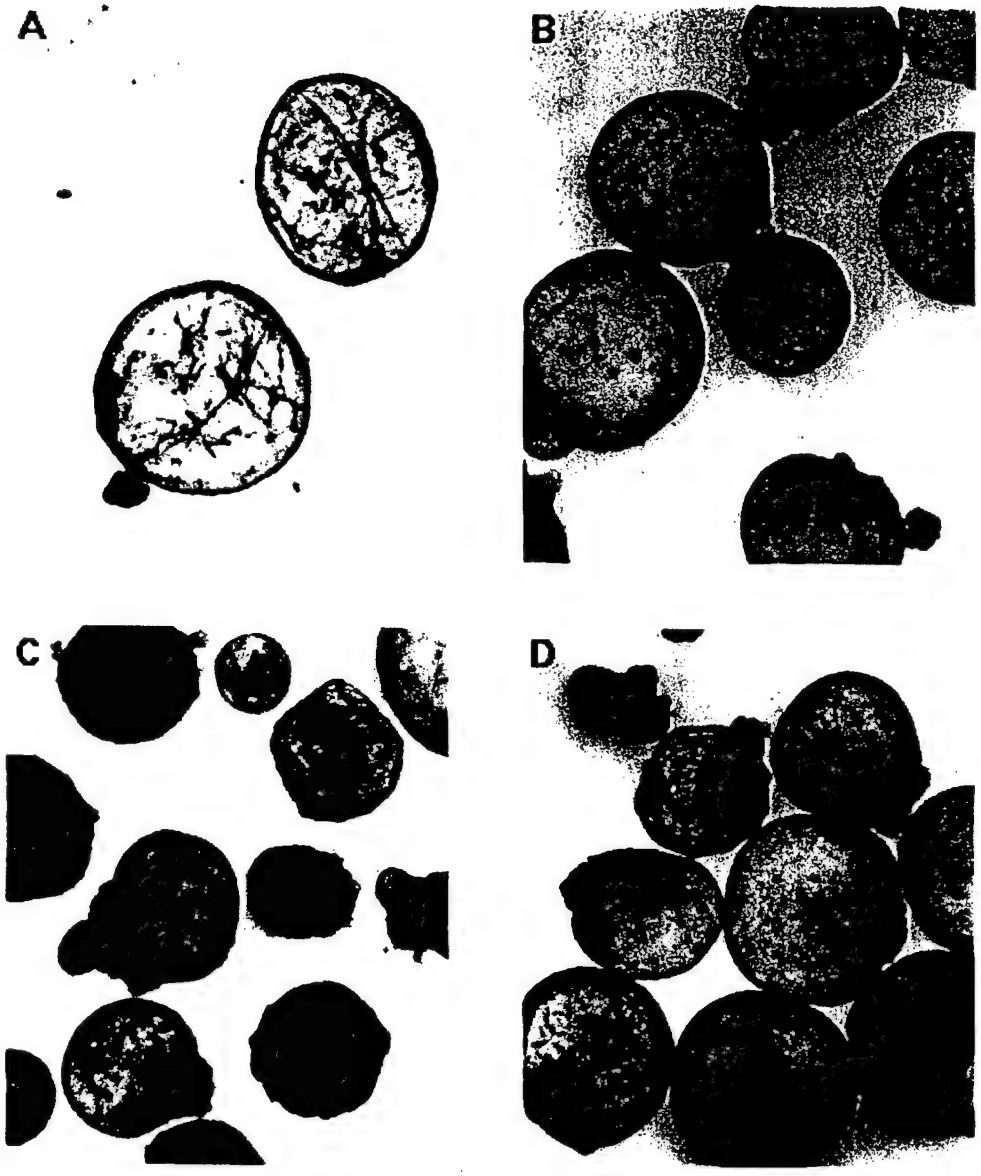
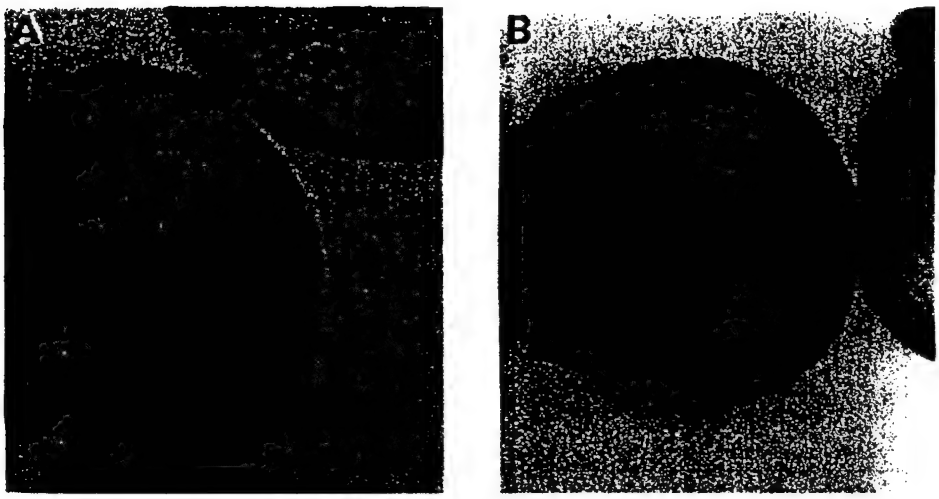

A
B
C
D

Fig. 4 Embryoid bodies (100X magnification) generated from either Tie+/- (A), 2G5R (B), 2G6 (C) or 1A12 (D) ES cells. The endothelial cells in embryoid bodies were visualized by β-galactosidase staining.


A
B
C
D

Discussion

The work described above is an ongoing collaboration with Jackie Ho in the laboratory of Dr. Janet Rossant (Toronto). The data presented here is preliminary, and further detailed analysis needs to be undertaken before definite conclusions can be made. Hence, I will limite the discussion of this chapter to a few short speculations.

It appears that expression of Notch4/int-3 disrupt vascular development, leading to a lethal phenotype. This phenotype may be the direct result of a defect in endothelial cells expressing Notch4/int-3, because the vascular structures that are observed in embryoid bodies experiments are not organized into vascular networks. Since the major blood vessels and the vessels in the brain initially develop in mutant embryos, our data suggest that Notch4/int-3 did not ablate vasculogenesis and angiogenesis. The presence of hematopoietic precursor cells in mutant embryos also suggest that Notch4/int-3 did not disrupt the cell fate decision that determines endothelial and hematopoitic fate (there is some evidence to believe that both these cells are derived from a common precursor "the hemangioblast"). The observed phenotype in mutant embryos may be due to disruptions of cell fate decisions, regulated by Notch, that are required for proper development of the vasculature in a three-dimensional organized structure.

The development of the vasculature is a dynamic process involving blood vessel construction, maintenance, remodeling and elimination. Further analysis of the Flk1/int-3 embryos may shed light on some of the developmental stages that occur during formation of the mouse embryonic vasculature.

Chapter 7

Discussion

In this chapter I will briefly summarize the data described in the previous four chapters. I will then propose a model for the biological function of Notch4.

The *int-3* oncogene was identified as a frequent target in Mouse Mammary Tumor Virus-induced mammary carcinomas, and encodes the intracellular domain of a novel mouse *Notch* gene. To investigate the role of the *int-3* proto-oncogene in mouse development and carcinogenesis, I isolated cDNA clones corresponding to the entire coding potential of the *int-3* proto-oncogene. I named this gene *Notch4* and reserve the *Notch4/int-3* nomenclature for references to the oncogenic form. The deduced amino acid sequence of Notch4 contains conserved motifs found in Notch proteins. Comparison of the coding potential of the *Notch4/int-3* gene to that of the *Notch4* suggests that loss of the extracellular domain of Notch4 leads to constitutive activation of this murine Notch protein. In situ hybridization and imunnohistochemical analysis revealed that *Notch4* transcripts are restricted to endothelial cells in embryonic and adult life.

Branching morphogenesis of the mouse mammary epithelial TAC-2 cell line was used as an assay to examine the role of Wnt, HGF, TGF-β and the Notch receptors in branching morphogenesis. Wnt-1 was found to induce the elongation and branching of epithelial tubules, like HGF and TGF-β2, and to strongly cooperate with either HGF or TGF-β2 in this activity. Wnt-1 displayed morphogenetic activity in TAC-2 cells as it induced branching even under conditions that normally promote cyst formation. The Notch4/int-3 mammary oncoprotein, an activated form of the Notch4 receptor, inhibited the branching morphogenesis normally induced by HGF and TGF-β2. The minimal domain within the Notch4/int-3 protein required to inhibit morphogenesis consists of the CBF-1 interaction domain and the cdc10 repeat domain. Co-expression of Wnt-1 and Notch4/int-3 demonstrates that Wnt-1 can overcome the Notch-mediated inhibition of ductal morphogenesis. These data suggest that Wnt and Notch signaling may play opposite

roles in mammary gland development, a finding consistent with the convergence of the wingless and Notch signaling pathways found in *Drosophila*.

Rat brain micro vessel endothelial cells (RBE4) were used as a model system to study the role of Notch4 and Jagged-1, a putative Notch ligand, in endothelial cell differentiation. Both Notch4/int-3 and Jagged-1 were able to induce cellular structures with morphological and biochemical properties of endothelial micro vessels. Ectopic expression of full length Notch4 did not have any discernible effect in RBE4 cells. Activation of the Notch signal transduction pathway was measured by the induction of endogenous *Notch4* and *Jagged-1* genes. The observed morphological changes to RBE4 cells correlated with endogenous *Notch4* and *Jagged-1* gene activation, demonstrating a link between Notch signaling and biological activity. These observations support a role for Notch signaling in cell fate determination during endothelial differentiation.

To study the *in vivo* role of Notch4 in endothelial cell development, homologous recombination in embryonic stem cells (ES) cells was used to drive expression of *Notch4/int-3* from the *Flk-1* promoter and regulatory elements to confer endothelial specific expression of *Notch4/int-3* starting at day 7 of embryonic development. To facilitate analysis of a putative phenotype, ES cells were used that were already programmed to express the β-galactosidase in the endothelium. Tetraploid aggregation experiments were used to generate mouse embryos that were completely derived from the ES cells. Mouse embryos that express Notch4/int-3 in the endothelium die around day 10 of embryonic development and display enlarged and collapsed blood vessels prior to death. To determine whether the observed phenotype is the result of an inherent defect in endothelial cells, embryoid bodies (EB) were generated from the ES cell lines to investigate the development of the endothelium. Vascular networks observed in EB that express Notch4/int-3 are disorganized and do not form the fine and homogenous vascular

networks which are observed in control embryoid bodies. These data thus suggest that Notch4/int-3 can disrupt the normal architectural patterns that are required for proper endothelial development.

The above described function of Notch4 in TAC2 mammary epithelial and RBE4 endothelial cells may seem, at first glance, to be opposite. In TAC2 cells Notch4/int-3 attenuates the HGF induced differentiation and morphogenesis, whereas in RBE4 cells Notch4/int-3 induces the differentiation of microvessel structures (similar to the bFGF activity). The apparent opposite biological effects observed in these two different tissue culture systems, can be explained if Notch4 regulates cell fate decisions in mammalian cells. I propose that Notch function in mammals is not restricted to blocking differentiation. Expression of the constitutive activated Notch4/int-3 may cause all cells to adopt a certain cell fate, and this cell fate may be characterized by the potential to differentiate into a specific phenotype. Depending on the presence or absence of factors required for the induction of differentiation, the observed phenotype of the particular cell fate instructed by Notch4/int-3, may either be characterized by differentiation or the lack thereof. For instance, expression of Notch4/int-3 in TAC2 cells may result in a cell fate that is characterized by the absence of the c-met receptor, hence these cells lose their responsiveness to HGF. However, it may be possible to differentiate TAC2 cells using other, in this case unknown, inducible factors. In RBE4 cells, expression of Notch4/int-3 may lead to secretion of bFGF, and since RBE4 cells express endogenous bFGF receptors, this autocrine loop would then result in the differentiation of RBE4 cells. Although the above described mechanisms are hypothetical and the exact mechanism leading to the opposite biological effects of Notch4/int-3 is not known, the data I have presented may support the notion that Notch4 regulates cell fate decisions. This conclusion supports the model that the function of *Notch* genes, regulation of cell fate decision, has been conserved through evolution.

Understanding of the biological function of Notch4 in mammary gland and endothelial cell development should be based on *in vivo* experiments. Tissue culture models may mimic some of the developmental processes observed *in vivo*, however, interpretations of the biological function based on results obtained from these systems require *in vivo* confirmation. For instance, where the TAC2 system demonstrates a Notch4/int-3 phenotype that is similar to the one observed phenotype in transgenic animal experiments, the RBE4 system demonstrates a somewhat different phenotype when compared to the phenotype observed in the Flk1/int-3 knock-in animal experiments.

There is no strong evidence for a role of Notch4 in mammary gland development since endogenous Notch4 protein in mammary epithelial cells *in vivo* has not been demonstrated. A detailed analysis of the Notch4 expression, either by in situ or by imunnohistochemistry, in the mouse mammary gland is indicated. However it seems unlikely that Notch4 is expressed in mammary epithelial cells, since our analysis as well as analysis by other investigators has demonstrated that Notch4 expression is restricted to endothelial cells. It would be surprising if the Notch4 protein, which is absent in all epithelial cells examined, would be specifically expressed in the epithelial cells of the mammary gland. It is possible that the murine *Notch1, 2, and 3* genes are expressed in the mammary gland and that the observed phenotype in transgenic mice overexpressing Notch4/int-3, represents the function of endogenous *Notch* genes in mammary gland development. Again, expression analysis of the different *Notch* genes in the mammary gland is indicated. So at this time, it appears that ectopic expression of Notch4/int-3 (which may or may not mimic endogenous Notch activity) leads to a failure of proper development of the mammary gland and the formation of tumors.

The combined observations of the phenotypes caused by expressing an activated Notch4 in the developing mammary gland and the developing endothelium, in conjunction with some of the Notch phenotypes observed in other organisms, may lead to a hypothetical model of the biological function of Notch4 *in vivo*. Regulation of cell fate decisions by Notch has been demonstrated to be required for the development of many tissues into distinct patterns. The role of Notch in establishing patterns becomes obvious when one looks at the effect of Notch mutations in a large group of equivalent cells. For instance in *Drosophila*, Notch regulates how the neuroectoderm develops into a checker board pattern that is characterized by certain cells adopting a neuronal fate, where other cells adopt an epidermal fate. In the chicken skin, Notch regulates how feathers develop in an evenly distributed pattern (Crowe et al., 1998), and there is evidence that Notch may play a similar role in the homologous mammalian skin appendix, hair (Kopan and Weintraub, 1993).

Although inductive signaling may play a role in the development of highly organized three-dimensional multi-cellular structures (such as the mammary gland tree and vascular networks), it is likely that cell fate decisions must occur within the population of these growing cells. Cell fate decisions could provide a mechanism to confer specific characteristics and function to subsets of cells, and thus allowing the structure to acquire complex developmental patterns (extent of branching, size of individual structures, even distribution). The mechanism that controls the organization of an equivalence group into subsets of cells (each with specific function and characteristics) must be innate to these cells. For instance, mammary epithelial and endothelial cells have the ability to develop into three-dimensional multi-cellular structures *in vitro*, where inductive factors are constant and evenly distributed. Thus, cell fate decisions within an equivalence group or a clonal population of cells could result in the establishment of different groups of cells, each with a specific cell fate and characteristics, which would enable the cells to organize

into a distinct three-dimensional pattern. *Notch* genes are likely candidates to regulate these processes, because *Notch* genes are implicated in pattern formation and, activated Notch4 abrogates the development of these three-dimensional structures. Thus, the phenotype observed in the mammary gland and the endothelium expressing activated Notch4, may be the result of the disruption of different populations of cells due to aberrant cell fate decisions. Analysis of markers in cells that are instructed to adopt a particular fate by an activated Notch, would provide an inroad towards the identification of the different mammary epithelial and endothelial cell types. These markers could then be used to identify the different cell types within the mammary gland and vasculature, and further our understanding of the development of these tissues.

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